STUDIES ON *LISTERIA MONOCYTOGENES.*

I. ISOLATION OF A MONOCYTOSIS-PRODUCING AGENT (MPA)

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Listeria monocytogenes[1] is the causative organism of a rare disease (more common in certain animals than in man) characterized by a monocytic leucocytosis, bacteraemia, necrosis of the liver and occasional meningo-encephalitic involvement. The organism has been isolated from a few cases of infectious mononucleosis, although the part, if any, it plays in the etiology of that disease is not yet clear (Nyfeldt, 1929; Pons and Julianelle, 1939; and Webb, 1943). Since the report of the first case of listeria meningitis in man by Burn (1934) only 15 cases have been reported (Burn, 1934, 1935; Schultz *et al.*, 1934; Gibson, 1935; Carey, 1936; Webb and Barber, 1937; Poston *et al.*, 1937; Wright and MacGregor, 1939; Porter and Hale, 1939; and Handelman *et al.*, 1946). Nine of the 15 patients died and six recovered. The first proven Australian case was recently described by the author (Stanley, 1948) and served as a stimulus for the investigations to be described here.

The unusual effect which infection by this bacterium produces on the haemopoietic system is shared by few organisms. McJunkin and Charlton (1919) observed marked increases in the number of circulating mononuclear cells in experimental tuberculosis in the guinea-pig. This observation has been repeatedly confirmed by other investigators and in other animals (Cunningham *et al.*, 1925; Morriss and Tan, 1927; Medlar and Kastlin, 1927), while in man active progressive tuberculosis is associated with a monocytosis (Blackfan and Diamond, 1929). The studies of Barber (1939) showed that members

[1] This organism was first submitted as a new species (*Bacterium monocytogenes*) in 1926 by Murray, Webb and Swann. These investigators isolated the organism from guinea-pigs and rabbits during an epizootic amongst laboratory animals at Cambridge. The same organism was recovered by Pirie (1927) from gerbilles in South Africa. He proposed the name *Listerella hepatolytica* for the organism. When he became acquainted with the prior claim of the English workers, the name *Listerella monocytogenes* was adopted. However, the generic name *Listerella* was disallowed in 1939 by the Third International Congress for Microbiology, on the ground that Jahn in 1906 had already used the name for a Myxotozoan. Pirie (1940) subsequently proposed that the name *Listeria* be substituted. Topley and Wilson (1946), however, emphasize the similarity of this organism to members of the genus *Erysipelothrix* and suggested the name *Erysipelothrix monocytogenes*. The relationship was investigated by Barber (1939), but until further evidence is available the author proposes to use the name *Listeria monocytogenes*.

of the genus *Erysipelothrix* possessed the power of stimulating monocyte production in rabbits. Monocytosis is also a characteristic of the following diseases:

(1) infectious mononucleosis (etiology unknown);
(2) some protozoan infections (malaria, kala-azar, amoebiasis);
(3) some cases of Banti's syndrome (etiology unknown);
(4) some cases of Hodgkin's disease (etiology unknown);
(5) monocytic leukaemia;
(6) some virus diseases (variola, typhus, yellow fever, dengue, etc.).

The monocytosis associated with virus infection is considered to be variable and small in amount (Stitt, Clay and Clough, 1938).

Because of the similarity of the haemopoietic response to infection with three different agents (listeria, mycobacteria, and the etiological agent of infectious mononucleosis), a study of the mechanism involved in the production of a monocytosis was undertaken.

It is the purpose of this paper to describe the preparation and some of the properties of a lipoid identified as a monocytosis producing agent (MPA) of *Listeria monocytogenes*.

Materials and Methods.

The strain of *Listeria monocytogenes* used was that recently isolated from the meninges of a fatal case of meningitis (Stanley, 1948).

Preparation of Bacterial Suspensions.

The culture medium consisted of neopeptone-heart-broth solidified with 1·5 p.c. powdered agar (Davis Gelatin Pty. Ltd.). The medium was distributed both in Roux bottles and roll-tubes, 80 ml. of medium being added to each Roux bottle and 15 ml. to each roll-tube (see Fig. 1). The agar was solidified in the roll-tubes by rotating them under a stream of cold water. These proved to be more satisfactory than the customary Roux bottles for the following reasons:

(1) they occupy less incubator space; (2) they use approximately one-eighth the volume of medium per unit surface area; (3) bacterial growth is very easily washed off the roll-tubes with the aid of a throat swab.

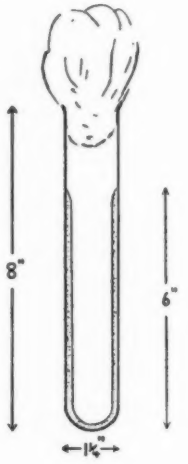

Fig. 1.

The surface of the agar in the Roux bottles and roll-tubes was inoculated with a small quantity of an 18-hour broth culture of the organism. Cultures were passaged in mice a few days before use, as it was found that continued subculture on blood agar resulted in decreased and variable yields of active material. The surface of the agar was inoculated with a 24-hour culture of the organism growing in neopeptone-heart-broth. The growth resulting after 24-hours' incubation at 37° C. was washed off in glass-distilled water.

Blood for total and differential leucocyte counts of rabbits was taken from the lateral vein of the right ear, while injections were made in the corresponding vein in the left ear. Bacterial extracts were tested for leucocytic response by injecting the saline suspensions prepared from them intravenously into rabbits. Total and differential leucocyte counts were performed every 24 hours after injection.

EXPERIMENTAL.

Isolation of MPA.

The MPA could be isolated from disrupted bacterial cells (in the dry state or in aqueous suspension) by extraction with lipoid solvents such as petroleum ether, chloroform or ether. At each stage of the extraction material was set aside for testing for the monocytic response in rabbits. The following schema shows the steps involved in a typical extraction using chloroform as the solvent.

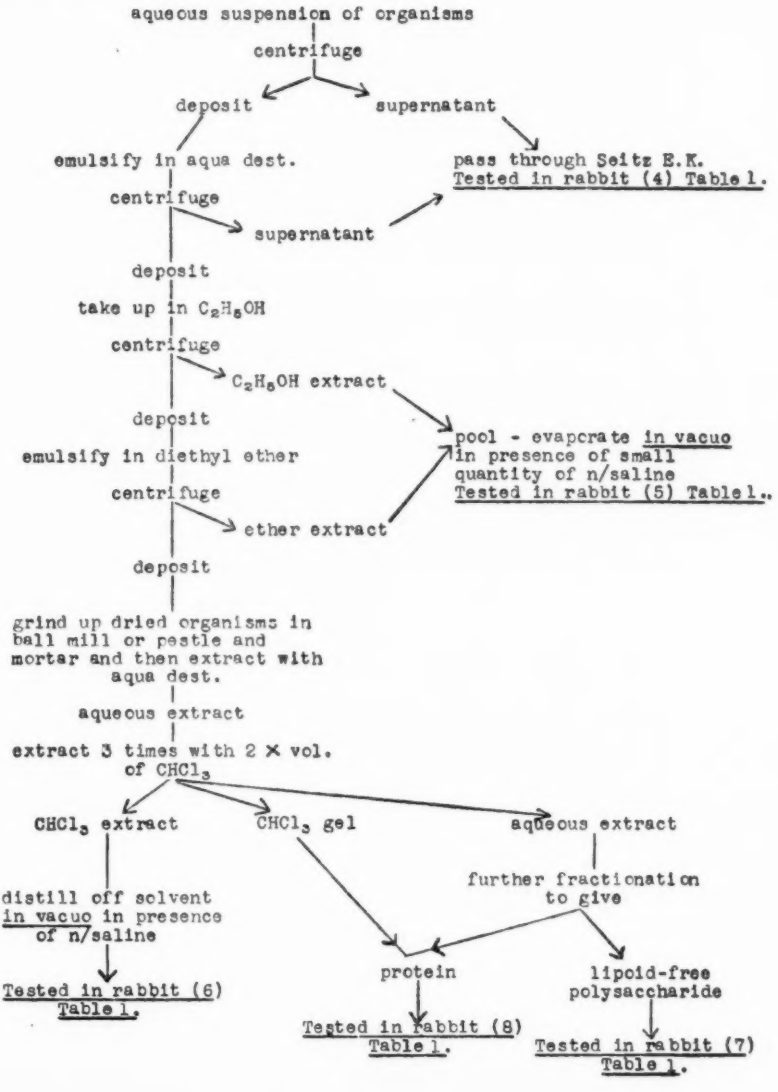

TABLE 1.

Rabbit number	Time of count	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*	
			Percentage	Absolute	Percentage	Absolute	Percentage	Absolute
(1)	before injection	11,200	77	8,600	20	2,300	3	300
	after 24 hours	10,800	20	2,200	76	8,200	4	400
0.5 ml. of 18-hour culture	" 48 "	15,200	56	8,500	37	5,600	7	1,100
	" 72 "	18,800	33	6,200	46	8,600	21	4,000
	" 96 "	18,300	40	7,300	36	6,600	24	4,400
	" 120 "	16,200	48	7,800	30	4,800	22	3,600
	" 144 "	18,600	48	8,900	34	6,300	18	3,400
(2)	before injection	10,300	58	6,000	38	3,900	4	400
	after 24 hours	12,000	23	2,800	73	8,700	4	500
0.3 ml. of a heat-killed suspension $20,000 \times 10^6$ orgs./ml.)	" 48 "	13,300	40	5,300	57	7,500	3	400
	" 72 "	10,000	54	5,400	39	3,900	7	700
	" 96 "	10,400	57	5,900	34	3,600	9	900
	" 120 "	14,000	49	6,900	43	6,000	8	1,100
	" 144 "	12,000	52	6,200	40	4,800	8	1,000
(3)	before injection	9,800	57	5,600	35	3,400	8	800
	after 24 hours	17,700	28	5,000	65	11,500	7	1,200
Seitz filtrate from an 18-hour liver infusion culture (2.0 ml.)	" 48 "	15,200	40	6,100	50	7,600	10	1,500
	" 72 "	10,700	51	5,400	37	4,000	12	1,300
	" 96 "	12,000	60	7,200	26	3,100	14	1,700
	" 120 "	13,100	48	6,300	44	5,800	8	1,000
	" 144 "	10,200	58	5,900	32	3,300	10	1,000
(4)	before injection	10,900	56	6,100	39	4,300	5	500
	after 24 hours	10,100	33	3,300	63	6,400	4	400
Sterile supernatant fluid from saline washings of organisms (1.5 ml.)	" 48 "	9,800	44	4,300	48	4,700	8	800
	" 72 "	10,800	53	5,700	36	3,900	11	1,200
	" 96 "	9,600	49	4,700	39	3,700	12	1,200
	" 120 "	11,000	52	5,700	35	3,900	13	1,400
	" 144 "	9,400	50	4,700	36	3,400	14	1,300

TABLE 1 (continued).

Rabbit number	Time of count	Total leucocytes*	Lymphocytes*		Polymorphonuclear cells*		Monocytes*	
			Percentage	Absolute	Percentage	Absolute	Percentage	Absolute
(5)	before injection	14,200	60	8,500	40	5,700	—	—
1.5 ml. of alcohol-ether washings (in saline)	after 24 hours	13,800	37	5,100	59	8,100	4	600
	" 48 "	13,800	48	6,600	42	5,800	10	1,400
	" 72 "	12,700	51	6,500	45	5,700	4	500
	" 96 "	11,000	48	5,300	46	5,100	6	600
	" 120 "	13,900	62	8,600	32	4,500	6	800
	" 144 "	12,000	59	7,100	33	4,000	8	900
(6)	before injection	12,800	76	9,700	22	2,800	2	300
1.5 ml. of chloroform extract	after 24 hours	11,600	65	7,500	23	2,700	12	1,400
	" 48 "	12,300	59	7,300	26	3,200	15	1,800
	" 72 "	13,700	65	8,900	13	1,800	22	3,000
	" 96 "	10,500	72	7,500	12	1,300	16	1,700
	" 120 "	11,800	68	8,000	26	3,100	6	700
	" 144 "	12,000	70	9,000	20	2,500	10	1,300
(7)	before injection	11,400	71	8,100	27	3,100	2	200
0.5 ml. of polysaccharide fraction (10 mg./ml.)	after 24 hours	29,000	26	7,500	72	20,900	2	600
	" 48 "	33,000	18	6,000	78	25,700	4	1,300
	" 72 "	18,000	30	5,400	67	12,100	3	500
	" 96 "	14,600	32	4,700	62	9,000	6	900
	" 120 "	12,800	32	4,100	64	8,200	4	500
	" 144 "	13,000	40	5,200	56	7,300	4	500
(8)	before injection	13,800	64	8,800	30	4,200	6	800
1.5 ml. Protein extract	after 24 hours	9,400	72	6,800	24	2,200	4	400
	" 48 "	11,000	76	8,300	18	2,000	6	700
	" 72 "	12,600	64	8,100	32	4,000	4	500
	" 96 "	10,200	61	6,200	36	3,700	3	300
	" 120 "	11,700	73	8,500	22	2,600	5	600
	" 144 "	12,600	70	8,800	25	3,200	5	600

* Per cubic millimetre.

The method outlined above may be varied by extracting the dried, ground, organisms directly with the lipoid solvent. Occasionally traces of polysaccharide and protein were found when the saline suspensions were tested for these compounds. They were removed from the MPA by extracting the saline suspension with ether or petroleum ether and then evaporating once more the ethereal solution *in vacuo* in the presence of saline. The leucocytic responses of extracts outlined in the above schema are recorded in Table 1. They may be compared with the response due to infection by the viable cell, that given by heat-killed cells (55° C.), and a sterile filtrate from a 24-hour broth culture.

It is seen that the $CHCl_3$ extract contains nearly all the active material and gives a haemopoietic response comparable with that produced by infection with the viable organism. Very little active material could be demonstrated in Seitz filtrates of cultures or from saline washings of living cells. The MPA is liberated more readily after disruption of the bacterial cell. Active material could also be removed by grinding up the organisms under chloroform, diethyl ether, or petroleum ether. The yield of lipoid material was greatest with petroleum ether, but the material was not as active biologically as the chloroform and ether extracts.

Chemical Properties.

The waxy material from the chloroform extract was relatively insoluble in water or saline, and slightly soluble in warm alcohol. It was extremely soluble in petroleum ether, chloroform and diethyl ether. Negative tests were obtained when testing for carbohydrate, protein and the nucleic acids.

Saline suspensions for biological work were obtained by evaporating the lipoid solution in the organic solvent *in vacuo* in the presence of normal saline. The resulting suspensions were invariably densely turbid. On standing in the cold the waxy material separated out and sometimes adhered to the glass walls or settled to the bottom of the container. The material obviously contained impurities.

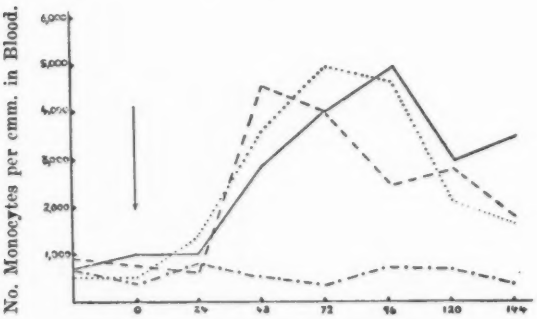

Fig. 2.

Serological Activity.

The MPA was non-antigenic for rabbits, and did not precipitate with serum prepared in the rabbit against the intact bacterial cell. Complement-fixation tests with the antiserum were negative. The monocytic response was obtained not only in normal rabbits, but also in those immunized against the intact cell. (See Fig. 2.)

Toxicity.

With the saline suspensions used no apparent toxic reactions could be demonstrated in mice and rabbits by intravenous, intramuscular or intradermal injections. Rabbits received up to 5 ml. of a densely turbid suspension injected intravenously. On the other hand the polysaccaride fraction was highly toxic for mice and rabbits. With doses smaller than the $LD._{50}$ the rabbits developed a neutrophil leucocytosis which was preceded by a leucopenia. If the amount of polysaccharide administered was equal to or greater than the $LD._{50}$ then the rabbits developed

a severe leucopenia and died within a few hours without producing a neutrophil leucocytosis.

MPA in the Organs of Infected Animals.

Five days after the intravenous injection of 0·5 ml. of an 18-hour broth culture into two rabbits the monocyte count was increased more than tenfold in each case. The rabbits appeared to be dying and were killed on the fifth day. Post-mortem examination revealed the characteristic multiple necrosis of the liver and spleen. The spleen was enlarged. Macroscopic examination of the lungs revealed no apparent abnormality. The livers, lungs and spleens were removed, cultured for listeria, and separately emulsified with twice their weight of sterile normal saline in a Waring blendor. The emulsified material was centrifuged and the clear supernatant fluids each divided into two portions, one of which was sterilized by filtration through Seitz E.K. filters and the other by heating at 55° C. for 45 minutes. Normal rabbit liver was subjected to the same process and all extracts tested by injecting 2·0 ml. intravenously into rabbits. The results recorded in graph form in Fig. 3 show that the necrotic liver produces a marked monocytosis.

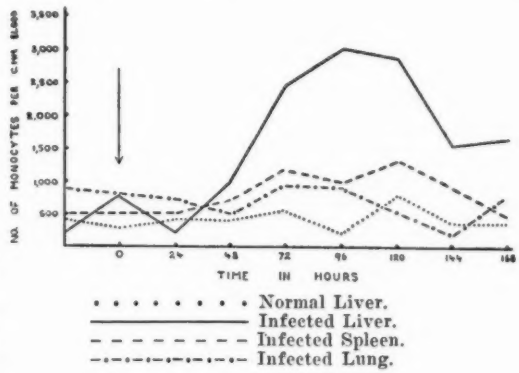

Fig. 3.

DISCUSSION.

The fact that the MPA of *List. monocytogenes* has proved to be part of the lipoid material of that organism is not surprising as it is known that some lipoids foreign to the experimental animal may act as a stimulus for the production of large monocytes. American workers claim that phthioic acid, a fatty acid fraction prepared from the phosphatides of *Myco. tuberculosis*, specifically stimulates the production of mononuclear cells in the tissues of man and animals (Cunningham *et al.*, 1925; Sabin and Doan, 1927; Anderson, 1927; Sabin *et al.*, 1930).

The monocytosis produced during experimental infection of animals with *Listeria monocytogenes* was of the same order of magnitude as that produced by intravenous injection of the MPA. However, the monocytosis of the infected animal persisted in the majority of experiments for a longer period of time than that induced by one injection of the MPA. This was probably due to the liberation of lipoid from the growing and dying organisms at the foci of infection in the animal.

Rabbits showing the greatest monocytic response to infection all showed, at post-mortem examination, necrosis of the liver that was more marked than in those animals whose haemopoietic response was poor. In the cases of non-fatal infection of rabbits, blood cultures were invariably sterile at all stages of

the disease in spite of a profound monocytosis. It was demonstrated that the necrotic liver contained material capable of causing a monocytic response while normal rabbit liver did not possess this property. Focal necroses of the liver and spleen are undoubtedly due to the local multiplication of listeria in these organs. The bacilli can be seen in sections and smears made from the necrotic liver and pure cultures are often obtained while blood cultures may be sterile. It is likely, therefore, that the MPA is liberated after multiplication and resulting lysis of bacterial cells has taken place and that this liberation of lipoid from liver, and to a lesser extent in the spleen or lung, may be responsible for the monocytosis of experimentally infected rabbits.

The MPA in the intact cell probably exists in fairly firm combination with a polysaccharide, as the polysaccharide prepared by alcohol precipitation before extraction of the MPA is capable of causing a monocytosis as well as a neutrophil leucocytosis, whereas that prepared after extraction of the MPA causes only a neutrophil leucocytosis. As lipoid-free protein extracts of listeria did not produce an increase in the number of circulating monocytes, it seems justifiable to conclude that the lipoid material itself is responsible for this effect.

The MPA could be isolated only in small amount from cultures that were completely rough (Scotland strain, Gibson, 1935) and very little from those strains producing intermediate type colonies. A decrease in yield of active material was noticed after subculture for some weeks on blood-agar. After passage of these supposedly rough strains through mice or rabbits an increased yield was obtained. Although the MPA has low toxicity it is present in relatively large amounts in those organisms which are highly virulent for mice and rabbits.

SUMMARY.

The isolation of a lipoid, designated as the monocytosis producing agent (MPA), from *Listeria monocytogenes* has been described. The MPA is serologically inactive and has low tissue toxicity, but is capable of producing a profound monocytosis both in the normal rabbit and the rabbit immunized against the intact bacterial cell.

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STUDIES ON *LISTERIA MONOCYTOGENES.*

II. A RÔLE OF LISTERIA IN INFECTIOUS MONONUCLEOSIS

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Infectious mononucleosis (glandular fever) is a disease that may assume several different clinical forms (Baldridge, Rohner and Hansmann, 1926; Tidy, 1934). There is still some doubt as to the nature of the etiological agent. The majority of evidence suggests that the disease is caused by a filtrable virus (van den Berghe and Liessens, 1939a and b; van den Berghe, Liessens and Kovacs, 1939; Wising, 1942; Burnet and Anderson, 1946; Anderson, 1947). The only noteworthy evidence which is antagonistic to the virus theory arose when Nyfeldt (1929, 1932) isolated *List. monocytogenes* from the blood of a case believed to be typical of infectious mononucleosis. Since that time other workers have succeeded in isolating listeria from other cases of the disease (Schmidt and Nyfeldt, 1938; Pons and Julianelle, 1939; Webb, 1943). These few cases may be too small to be significant, yet the results of investigation on the virus origin of the disease are by no means conclusive.

The following studies were carried out to determine whether several strains of *Listeria monocytogenes* could play any possible rôle in the etiology of infectious mononucleosis.

Materials and Methods.

Blood cultures from patients were made in liver infusion, neopeptone-heart-broth and cooked-meat medium. They were incubated at 37° C. for 3 weeks and before being discarded as sterile were inoculated on to blood agar plates.

Blood cultures from rabbits were carried out by taking 0·2 ml. of blood (in a sterile pipette) from the lateral vein of the left ear and immediately adding the blood to 2·0 ml. of nutrient agar (cooled to 47° C.) in 6 in. × ¾ in. sterile test tubes. The agar was sucked up and down the pipette four times to remove blood adhering to the glass. The agar was then solidified on the walls of the test tube by rotating the tube under a stream of cold water. The rabbits' blood-agar roll tubes were incubated at 37° C. in an inverted position for 48 hours. The number of colonies developing could then be counted and an approximation of the number of viable cells per ml. of blood obtained.

Tests for sheep red-cell agglutinins were carried out by the modification of the Paul-Bunnell technique described by Barrett (1941).

TABLE 1.

	Patient number	Date of collection of blood	Blood culture	Paul-Bunnell titre	Listeria agglutination titre		Complement fixation with MPA	Agglutination titres.							
					Aust. strain	Carey's strain		S. typhi (H)	S. typhi (O)	S. paratyphi A (H)	S. paratyphi A (O)	S. paratyphi B (H)	S. paratyphi B (O)	Proteus OX 19	Br. abortus
Infectious mononucleosis cases	1	22-3-48	sterile	>1,000	256	—	—	<20	—	<20	—	<20	—	<20	<20
	2*	22-3-48	sterile	64	<4	—	—	20	—	40	—	80	—	<20	<20
	3	23-3-48	sterile	32	<4	—	—	<20	—	<20	—	<20	—	<20	<20
		31-3-48	sterile	256	128	—	—	<20	—	<20	—	<20	—	<20	<20
	4	26-3-48	sterile	256	<4	—	—	<20	—	<20	—	<20	—	<20	<20
		8-4-48	sterile	256	4	—	—	<20	—	<20	—	<20	—	<20	<20
	5	31-3-48	—	32	<4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	6	19-4-48	sterile	128	8	<4	negative	<20	—	<20	—	<20	—	<20	<20
	7	19-4-48	sterile	512	32	32	negative	<20	—	<20	—	<20	—	<20	<20
	8	29-4-48	sterile	1,000	16	64	negative	<20	—	<20	—	<20	—	<20	<20
		26-5-48	—	128	4	8	negative	<20	—	<20	—	<20	—	<20	<20
	9*	19-5-48	sterile	<16	<4	<4	negative	20	—	80	—	40	—	<20	<20
	10	26-5-48	haem. staphs.	128	4	4	negative	<20	—	<20	—	<20	—	<20	<20
	11	28-5-48	—	64	<4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	12	31-5-48	sterile	1,000	4	<4	negative	<20	—	<20	—	<20	—	<20	<20
		9-6-48	—	1,000	<4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	13	1-6-48	sterile	32	4	<4	negative	<20	—	<20	—	40	—	<20	<20
		5-6-48	sterile	32	4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	14	7-6-48	sterile	<16	<4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	15	7-6-48	sterile	64	16	<4	negative	<20	—	<20	—	<20	—	<20	<20
	16	7-6-48	sterile	64	16	<4	negative	<20	—	<20	—	<20	—	<20	<20
		15-6-48	—	512	64	<4	negative	<20	—	<20	—	<20	—	<20	<20
	17	8-6-48	*Staph. albus*	<16	<4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	18	10-6-48	—	32	4	<4	negative	<20	—	<20	—	<20	—	<20	<20
	19	15-6-48	sterile	64	4	4	negative	<20	—	<20	—	<20	—	<20	<20
	20	21-6-48	sterile	>1,000	64	16	negative	<20	—	<20	—	<20	—	<20	<20

TABLE 1 (continued).

	Patient number	Date of collection of blood	Blood culture	Paul-Bunnell titre	Listeria agglutination titre		Complement fixation with MPA	Agglutination titres.							
					Aust. strain	Carey's strain		S. typhi (H)	S. typhi (O)	S. paratyphi A (H)	S. paratyphi A (O)	S. paratyphi B (H)	S. paratyphi B (O)	Proteus OX 19	Br. abortus
Control cases	21	26-3-48	sterile	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	22	5-4-48	—	—	<4	—	—	40	<20	<20	<20	<20	20	<20	<20
	23†	7-4-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	1,280	<20
	24	7-4-48	sterile	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	25*	8-4-48	sterile	—	4	—	—	40	<20	40	<20	40	20	<20	<20
	26	8-4-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	27	12-4-48	sterile	—	4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	28	12-4-48	—	—	<4	<4	—	<20	<20	<20	<20	<20	20	<20	<20
	29	13-4-48	—	—	<4	<4	—	<20	<20	40	<20	20	20	<20	<20
	30	14-4-48	sterile	—	<4	<4	—	<20	<20	<20	<20	<20	20	<20	<20
	31*	19-4-48	—	—	<4	<4	—	<20	<20	160	<20	40	20	<20	<20
	32*	20-4-48	—	—	<4	<4	—	<20	<20	<20	<20	80	20	<20	<20
	33	21-4-48	—	—	4	<4	—	<20	<20	<20	<20	40	20	<20	<20
	34	26-4-48	—	—	8	<4	—	<20	<20	<20	<20	<20	20	<20	<20
	35	27-4-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	36	30-4-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	37	4-5-48	—	—	4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	38	5-5-48	sterile	—	8	—	—	<20	<20	<20	<20	<20	20	<20	<20
	39*	17-5-48	sterile	—	4	—	—	20	<20	80	40	40	20	<20	<20
	40	17-5-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	41	25-5-48	sterile	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	42	7-6-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	43	15-6-48	sterile	—	4	—	—	<20	<20	<20	<20	<20	20	<20	<20
	44	18-6-48	—	—	<4	—	—	<20	<20	<20	<20	<20	20	<20	<20

— = test not performed. * = patient had received T.A.B. vaccine. † = case diagnosed as typhus fever.

Three strains of listeria were used, viz.:

1. The Australian strain, isolated from a fatal case of meningitis (Stanley, 1948).
2. An English strain (BCh-G), isolated from a case of infectious mononucleosis (Webb, 1943).
3. An American strain, isolated from a case of meningitis (Carey, 1936).

Twenty consecutive cases of infectious mononucleosis occurring between March and June, 1948, were selected for study.

EXPERIMENTAL.

Examination of Infectious Mononucleosis Cases.

These investigations were designed to determine (1) whether any relationship existed between Paul-Bunnell titre and the presence of agglutinins for listeria; (2) whether listeria could be isolated from the blood of patients after the diagnosis of infectious mononucleosis had been made by haematological and clinical means; (3) whether the presence of listeria agglutinins was due to nonspecific stimulation of antibodies; and (4) whether any serological relationship existed between MPA (Stanley, 1949) and the patient's serum.

Blood was collected from patients diagnosed clinically and haematologically as cases of infectious mononucleosis and

(1) cultured for organisms;
(2) tested for agglutinins to sheep red-cells by the modified method of Paul and Bunnell;
(3) tested for listeria agglutinins;
(4) tested for complement-fixing antibodies to listeria MPA, and;
(5) tested for agglutinins to *Proteus* OX 19, *Br. abortus* and members of the typhoid-paratyphoid group.

The results recorded in Table 1 do not include tests with the English (BCh-G) strain as this was not available at the time. A control group of sera was examined from patients whose blood had been sent in for routine agglutination tests, covering the same period of time as the test cases (March–June, 1948). Blood cultures were done on only some of these patients. Most of them were provisionally diagnosed as "pyrexia of unknown origin", some as virus pneumonia, an occasional typhus fever, etc. The sera of this control group were not tested against the MPA.

With one exception (case 8), the agglutination titres against listeria of sera from infectious mononucleosis cases were greater for the Australian strain than for Carey's strain. These two strains were shown to belong to different antigenic types (Stanley, 1948). In the control series the highest recorded titre against listeria was 1/8. A significant titre was, therefore, assumed to be 1/16 or greater. In no case in the test series was a significant titre obtained without the Paul-Bunnell titre being also significant (1/64 or greater). Seven of the twenty infectious mononucleosis cases gave significant Paul-Bunnell tests and significant listeria agglutinations, whilst six gave positive Paul-Bunnell tests and insignificant listeria agglutinations. The remaining seven cases gave insignificant titres for both tests. Complement-fixation tests with MPA were negative throughout, although this was more or less expected as the MPA was shown to be serologically inactive (Stanley, 1949). There was no evidence that antibodies to *Proteus* OX 19, *Br. abortus*, *S. typhi*, *S. paratyphi A* and *S. paratyphi B* were nonspecifically stimulated.

TABLE 2.

0·1 ml. of 18 hour culture	Time of count	Total leucocytes[1]	Lymphocytes[1]		Polymorphonuclear cells[1]		Monocytes[1]		Blood culture	Paul-Bunnell titre	Titre of serum against whole organisms
			Percentage	Absolute	Percentage	Absolute	Percentage	Absolute			
Intravenous inoculation	Before injection	10,000	65	6,500	31	3,100	4	400	sterile	<16	<4
	After 3½ hours	7,300	48	3,500	49	3,600	3	200	approx. 50 orgs./ml.	—	—
	After 22 hours	10,000	28	2,800	70	7,000	2	200	sterile	<16	—
	After 26½ hours	11,500	42	4,800	54	6,200	4	500	sterile	—	—
	After 46 hours	11,000	52	5,700	38	4,200	10	1,100	sterile	—	<4
	After 79 hours	12,200	44	5,400	35	4,200	21	2,600	sterile	<16	—
	After 84 hours	11,900	55	6,500	21	2,500	24	2,900	sterile	—	—
	After 108 hours	13,300	39	5,200	43	5,700	18	2,400	sterile	—	—
	After 132 hours	17,800	47	8,400	39	6,900	14	2,500	sterile	<16	32
	After 156 hours	13,200	50	6,600	42	5,500	8	1,100	sterile	—	—
	After 180 hours	18,000	68	12,200	22	4,000	10	1,800	sterile	—	—
	After 204 hours	12,000	50	6,000	42	5,000	8	1,000	sterile	<16	256
Intramuscular inoculation	Before injection	9,600	55	5,300	41	3,900	4	400	sterile	<16	<4
	After 3½ hours	12,000	35	4,200	57	6,800	8	1,000	sterile	—	—
	After 22 hours	13,600	21	2,900	76	10,300	3	400	sterile	—	—
	After 26½ hours	12,000	20	2,400	75	9,000	5	600	sterile	—	—
	After 46 hours	11,000	36	3,900	58	6,400	6	700	sterile	<16	<4
	After 79 hours	10,700	33	3,500	52	5,600	15	1,600	sterile	—	—
	After 84 hours	14,700	15	2,200	60	8,800	25	3,700	sterile	—	—
	After 108 hours	14,800	33	4,900	47	6,900	20	3,000	sterile	<16	—
	After 132 hours	13,600	47	6,400	36	4,900	17	2,300	sterile	—	4
	After 156 hours	10,600	52	5,500	38	4,000	10	1,100	sterile	—	—
	After 180 hours	13,800	43	5,900	46	6,400	11	1,500	sterile	—	—
	After 204 hours	16,000	29	4,700	62	9,900	9	1,400	sterile	<16	16
Intraperitoneal inoculation	Before injection	10,700	74	7,900	24	2,600	2	200	sterile	<16	<4
	After 3½ hours	13,200	65	8,600	31	4,100	4	500	sterile	—	—
	After 22 hours	19,200	41	7,900	51	9,800	8	1,500	sterile	—	—
	After 26½ hours	13,800	42	5,800	53	7,300	5	700	sterile	—	—
	After 46 hours	16,000	42	6,700	56	9,000	2	300	sterile	<16	<4
	After 79 hours	18,600	45	8,400	47	8,700	8	1,500	sterile	—	—
	After 84 hours	17,200	26	4,500	56	9,600	18	3,100	sterile	—	—
	After 108 hours	18,800	30	5,700	56	10,500	14	2,600	sterile	—	—
	After 132 hours	24,200	25	6,000	56	13,600	19	4,600	sterile	<16	8
	After 156 hours	38,800	23	8,900	57	22,100	20	7,800	sterile	—	—
	After 180 hours	17,200	33	5,700	55	9,400	12	2,100	sterile	—	—
	After 204 hours	23,200	51	11,800	40	9,300	9	2,100	sterile	<16	32

[1] Per cubic millimeter. — = test not performed.

Examination of Rabbits after Inoculation of Viable and Dead Listeria and Listeria Fractions.

There is no record of any investigator obtaining significant Paul-Bunnell titres in rabbits after the injection of living or dead listeria. Experiments carried out with the Australian strain confirm this finding. One ml. of an 18-hour broth culture was injected into each of 3 rabbits by the intravenous, intramuscular and intraperitoneal route respectively. Total and differential leucocyte counts, blood cultures, Paul-Bunnell tests and titrations for the presence of listeria agglutinins were carried out at appropriate intervals. The results recorded in Table 2 are those obtained with *List. monocytogenes* (Aust.). Similar results have been obtained both with *List. monocytogenes* (BCh-G) and *List. monocytogenes* (Carey).

A profound monocytosis developed in each case and the Paul-Bunnell titre was insignificant from the beginning to the end of the experiment. The antibody titre was highest in the intravenously inoculated rabbit (1/256) and lowest in the intramuscularly-inoculated animal (1/16). After intravenous inoculation of a culture, the organisms rapidly disappeared from the bloodstream (within 5 hours). No other positive blood cultures were obtained. However, in animals dead or dying from an infection, blood cultures were mostly positive.

Listeria lipo-polysaccharide, heat-killed cells and MPA were prepared from both the Australian and the BCh-G strain. Each preparation was repeatedly injected into rabbits over a period of 6 weeks, injections being made at 7-day intervals. The sera obtained from such rabbits did not agglutinate sheep red-cells although antibodies were developed against the intact bacterial cell and the polysaccharide, but not against the MPA, and each rabbit developed a monocytosis. The MPA and the polysaccharide fractions *per se* were unable to cause the agglutination of sheep red-cells. Negative tests were also obtained with necrotic liver extract (Stanley, 1949) and with sera taken from rabbits receiving injections of necrotic liver extract.

Tests with Human Red-Cells treated with the Virus of Newcastle Disease.

Through the courtesy of Prof. F. M. Burnet, rabbit sera containing listeria agglutinins were tested against human red-cells treated with Newcastle disease virus. Several human sera whose titre against NDV-H cells was known were also made available for testing for listeria agglutinins.

TABLE 3.

Serum	Paul-Bunnell titre	Titre agaist listeria monocytogenes (Aust.)	Titre against NDV-H red-cells
Rabbit A	<16	5,000	100
Rabbit B	<16	20,000	50
Rabbit C	<16	320	<25
Human serum A	30	32	1,000
Human serum B	640	16	800
Human serum C	320	16	640
Human serum D	2,000	<4	10
Human serum E	30	<4	2,400
Human serum F	100	<4	<25

Rabbit serum A = antiserum prepared in the rabbit against a heat-killed suspension of *List. monocytogenes* (Aust.). Serum heated in water bath at 55° C. for 20 mins.

Rabbit serum B = same as A + 0·25 p.c. phenol.

Rabbit serum C = antiserum prepared in the rabbit against the living organism. Serum heated in water bath at 55° C. for 30 mins.

The results shown in Table 3 indicate that the titres of the listeria antisera when used against NDV-H cells were below the level (1:120) considered to be pathognostic of infectious mononucleosis. Human serum A represents the only case in which a patient with a negative Paul-Bunnell test has given a significant listeria agglutination titre. There appeared to be no correlation between the titre against NDV-H cells and the titre against *List. monocytogenes*.

Absorption of Infectious Mononucleosis Sera with Listeria Suspensions.

Sera giving positive Paul-Bunnell tests were divided into two portions. One was mixed with an equal volume of a saline suspension ($20{,}000 \times 10^6$ orgs./ml.) of *Listeria monocytogenes* (Aust.) and allowed to stand at 4° C. from 18–24 hours. The other was mixed with an equal volume of saline and allowed to stand at the same temperature for the same time. After centrifugation to remove the organisms, Paul-Bunnell and listeria agglutination tests were carried out. The results recorded in Table 4 indicate that there was no significant fall in the Paul-Bunnell titre after absorption with listeria suspensions.

TABLE 4.

Patient number	Titre before absorption		Titre after absorption	
	Paul-Bunnell	listeria	Paul-Bunnell	listeria
3	256	128	256	<4
7	512	32	512	<4
12	1,000	4	512	<4
16	512	64	256	<4
20	1,000	64	1,000	<4

DISCUSSION.

In cases of infectious mononucleosis it is obvious that Paul-Bunnell titres are related neither to the severity of the disease nor to the degree of monocytosis[1]. The percentage of significant titres recorded in different series in the literature has varied from 0 to 100. In the United Kingdom two outbreaks occurred, affecting in all 278 people, in which the Paul-Bunnell tests were almost uniformly negative (Shafer and Weir, 1943; McFarlan and MacFarlane, 1943). In fact, in the outbreak described by McFarlan and MacFarlane, not one of the 78 patients gave a positive Paul-Bunnell reaction. These reports, are, however, exceptional. It is usual to find that approximately 30 p.c. of cases that are clinically and haematologically diagnosed as infectious mononucleosis give negative Paul-Bunnell tests (Kaufmann, 1944; Press *et al.*, 1945; Kruger *et al.*, 1945). Especially is this so where great attention has been paid to the carrying out and interpretation of the Paul-Bunnell test by absorption techniques. In the series of cases described in this paper 13 out of 20 (65 p.c.) gave positive Paul-Bunnell tests. Of these 13 cases, 7 (35 p.c.) gave significant listeria

[1] There is some doubt as to the exact nature of the so-called 'monocytosis' in this disease. It is now common practice to refer to these cells as 'atypical' or 'pathological lymphocytes'.

agglutinations. It is possible, therefore, to classify these cases into three serological groups, viz.:

	Paul-Bunnell test	Listeria agglutination	
A	+	+	35 p.c.
B	+	—	35 p.c.
C	—	—	30 p.c.

What, then, is the significance of a positive listeria agglutination in infectious mononucleosis? The studies of Janeway and Dammin (1941) are not as convincing as those of other workers in this field as they found no significant trend upward or downward in listeria agglutination during the course of the disease. On the contrary, the results in Table 1 show that in case 3 both the Paul-Bunnell and the listeria agglutination titre increased over a period of 8 days (Paul-Bunnell $\frac{1}{32}$ to $\frac{1}{256}$; listeria $\frac{1}{4}$ to $\frac{1}{128}$). In case 8, a decrease in titres was recorded over a period of 4 weeks (Paul-Bunnell $\frac{1}{1000}$ to $\frac{1}{128}$; listeria (Aust.) $\frac{1}{16}$ to $\frac{1}{4}$; listeria (Carey) $\frac{1}{64}$ to $\frac{1}{8}$). The latter patient had been discharged from hospital 20 days before the second sample of serum was collected. These results are in agreement with those of Julianelle (1940) in U.S.A. and Webb (1943) in Great Britain. The former investigator found that 13 of 28 patients (46 p.c.) gave listeria titres varying from $\frac{1}{20}$ to $\frac{1}{160}$. Webb found that 6 out of 9 patients gave titres varying from $\frac{1}{125}$ to $\frac{1}{500}$ and in one case demonstrated a rise in titre from $\frac{1}{25}$ to $\frac{1}{125}$ over a period of 11 days.

An observation worthy of note is that the Australian strain of *List. monocytogenes* was (with one exception) agglutinated to higher titre than the American strain.

Evidence is presented in this paper to show that nonspecific stimulation of listeria agglutinins in humans is not likely to be responsible for the positive results obtained. It was also shown that rabbits exhibiting a marked monocytosis after intramuscular and intraperitoneal injection of sufficiently small numbers of listeria to produce a non-fatal infection developed listeria agglutinins to titres of only $\frac{1}{16}$ and $\frac{1}{32}$ respectively (Table 2). The positive listeria agglutinins in humans do, therefore, assume a significance which should not be overlooked.

The blood of 17 of the 20 patients was cultured for organisms and *List. monocytogenes* was not isolated from any one case. If infection with listeria is responsible for the clinical, haematological and serological findings of the 7 patients giving significant agglutination reactions, why was the organism not isolated from the blood culture of any one of them? There may be several explanations:

(1) Infectious mononucleosis is a benign disease, most cases being diagnosed late in the course of the disease when the Paul-Bunnell test is positive and there is an increase in the number of atypical lymphocytes. It is likely, therefore, that bacteraemia at this stage is rare.

(2) In experimental listeria infection in rabbits it was shown that invasion of the blood stream occurred just prior to death, while in the case of mild, non-fatal infections it could not be demonstrated at all by the methods used. Blood cultures were sterile during the entire period of antibody production and the stimulation of monocytes.

(3) In rabbits, it is believed that the monocytosis is due to a lipoid liberated from a focus of infection such as the liver or spleen and not from organisms in the blood stream (Stanley, 1949). Positive blood cultures would, therefore, be more likely to occur early in the disease before the production of a monocytosis and before agglutinins are detectable.

The serious drawback in postulating any relationship between infectious mononucleosis and *List. monocytogenes* is the inability of the organism to stimulate the formation of sheep red-cell agglutinins in the rabbit (Kolmer, 1939). This observation was confirmed and extended to show that the polysaccharide, protein and lipoid fractions of *List. monocytogenes* could neither agglutinate sheep red-cells themselves, nor stimulate the production of sheep red-cell agglutinins in the rabbit. It is also unlikely that the antibody represents a response to some substance liberated from tissue cells during infection with listeria (Stanley, 1949). It was not possible to reduce the Paul-Bunnell titre of sera from infectious mononucleosis cases by absorption with listeria suspensions. These observations indicate that the Paul-Bunnell antibody is distinct from listeria antibody in spite of the correlation with human cases, observed in Table 1.

It is likely, therefore, that in dealing with so-called "infectious mononucleosis," we are not dealing with one disease, but with two or more entities almost indistinguishable clinically and haematologically (Schultz, 1930 and Horing, 1933). Even if the clinical findings simulate so many other disease syndromes it is at least possible to classify cases serologically into the 3 groups suggested. Group A may be those cases caused by infection with listeria and the other groups may be due to infection with a virus, both agents probably being distinct from the agent causing sheep cell agglutination.

It seems that the rôle of listeria in infectious mononucleosis must await further study before a definite answer can be obtained. It may be possible, in future, to correlate more closely the clinical manifestations of the disease with the serological findings, especially if these include tests with NDV-H cells and listeria suspensions.

SUMMARY.

The rôle of *Listeria monocytogenes* in infectious mononucleosis was studied in a series of 20 consecutive cases. These could be divided into 3 serological groups depending on the titres obtained in the modified Paul-Bunnell test and the listeria agglutination test. Experiments carried out with listeria fractions, including the monocytosis producing agent, gave negative results. It is concluded that some cases of infectious mononucleosis may be due to infection with *Listeria monocytogenes*.

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DECOMPOSITION OF OXALACETIC ACID BY METAL COMPOUNDS

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In spite of its importance in the intermediary metabolism of most living cells, the chemistry of oxalacetic acid (O.A.A.) has not been investigated in detail. The chemical literature deals mainly with its tautomerism (e.g. Hantzsch, 1915; Bredt, 1937), the action of aniline citrate and certain other amines on O.A.A. (Edson, 1935; Shishova, 1947; Ostern, 1933), and isolated observations such as Raman spectra (Kohlrausch and Pongratz, 1934).

The catalytic effect of polyvalent cations on the β-ketonic decomposition of O.A.A. has also been observed. Krebs (1942) described the effect of salts on this process, and similar work was recently carried out in this laboratory (Nossal, 1948). Data on the effect of H ion concentration on this "spontaneous decarboxylation" (as it has been referred to) are cited in both these communications.

Ochoa (1948) demonstrated the formation of highly unstable complexes between Al^{+++} and O.A.A. (as well as oxalsuccinate), which possess characteristic ultraviolet absorption spectra.

The decarboxylation of O.A.A. by cell extracts has been studied by many workers, and the catalysts have been shown to be heat-labile, protein in nature, and requiring Mn^{++} or Mg^{++} for activity. However, apart from the fact that the catalyzed reaction is of the first order, no results whatever concerning optimum pH and the effects of temperature, salt concentration, buffer composition etc. were recorded.

Recently, a decarboxylation of O.A.A. by blood was reported from this laboratory (Nossal, 1948; Nossal and Kerr, 1948). The variation of the activity of blood preparations with pH was so unusual that it was decided to investigate, in detail, the catalysis of O.A.A. decomposition by inorganic agents.

The interpretation of the effects cited in this work may have some bearing on the elucidation of the decarboxylation mechanism of O.A.A. by systems derived from living tissues.

Methods.

The decomposition of O.A.A. was followed by measuring CO_2 production in standard Warburg equipment. The total amount of O.A.A. present at the commencement of each

experiment was estimated by the aniline citrate method (Edson, 1935). The total volume/cup was maintained at 3·0 ml. The medium used for all solutions was distilled water redistilled from an all-glass apparatus. Measurements of pH were carried out with standard glass-electrode equipment. Metallic ions were made up in stock solutions so that 0·2 ml. would give the desired concentration when diluted to 3·0 ml.

Substrate concentration was such that the total theoretical CO_2 output should have been ca. 400 μl., had no decomposition of O.A.A. occurred in the period between making up the solution and the commencement of the experiment.

The following buffers were used in this work: HAc-NaAc ("acetate"), KH_2PO_4-Na_2HPO_4 ("phosphate"), disodium citrate-NaOH and disodium citrate-HCl ("citrate"), citric acid-Na_2HPO_4 ("citrate-phosphate"), potassium biphthalate-HCl ("phthalate") and KCl-HCl ("chloride").

RESULTS.

I. O.A.A. Decomposition without Metal Catalysts.

Stability of O.A.A. Solutions.

Other workers in this field seem to have partly neutralized the O.A.A. solutions for each experiment (Umbreit *et al.*, 1946). Initially, this procedure was adopted here, but in experiments at low H ion concentrations it became necessary to use unneutralized O.A.A., so that addition of substrate would not cause the system to shift towards neutrality. It was found that, although the same amount of substrate was used, the total CO_2 as determined by aniline citrate, was always significantly higher with unneutralized than with partly neutralized samples. Furthermore, it was found that unneutralized samples could be stored on ice for up to six hours after being made up without significant decrease in concentration, which was definitely not possible with partly neutralized solutions.

It was, therefore, concluded that sodium ions, or some impurity in the NaOH, accelerated the spontaneous decomposition of the substrate, and unneutralized solutions were used wherever possible.

TABLE 1.

The effect of temperature on O.A.A. decomposition.

Conditions: Each cup contains 0·2 ml. O.A.A. in the side-bulb, and 2 M acetate buffer in the main compartment.

Total volume, 3·0 ml. Experimental period, 60 mins. at 28° C., 30 mins. at 38° C.

pH	Total CO_2 outputs (μl.)		
	At 28° C.	At 38° C.	Theoretical CO_2*
6·5	48	92	390
5·0	65	130	390
3·5	94	167	390

* CO_2 evolved by action of aniline citrate.

The Effect of Temperature.

The spontaneous decomposition of O.A.A. in 2 M acetate buffers at varying pH values was examined simultaneously at 28° C. and 38° C. Such an experiment is shown in Table 1, from which it can be seen that, although the process is less rapid at the lower temperature, the increases in decomposition rate as the pH is lowered from 6·5 to 3·5 are of the same order in both cases.

Effect of Buffer Concentration.

Increases in the molarity of the buffer at any one pH are definitely accompanied by increases in the rate of decarboxylation, as shown in Fig. 1.

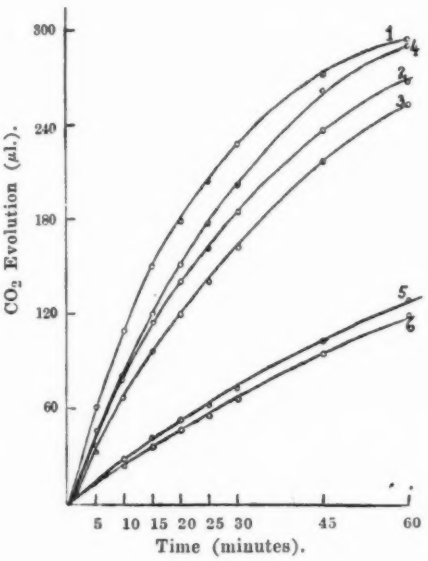

Fig 1.

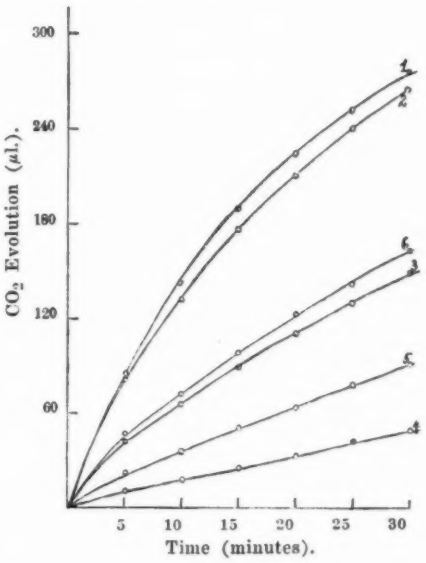

Fig. 2.

Fig. 1. Effect of buffer concentration on O.A.A. decomposition.

All cups contain 0·2 ml. substrate in the side-bulb, and 2·8 ml. of the appropriate acetate buffer in the main compartment.

Curve 1: 10 M buffer, pH 3·5
Curve 2: 2 M buffer, pH 3·5.
Curve 3: 0·2 M buffer, pH 3·5.
Curve 4: 10 M buffer, pH 5·0.
Curve 5: 2 M buffer, pH 5·0.
Curve 6: 0·2 M buffer, pH 5·0.

Fig. 2. Comparison of O.A.A. decomposition in 2 M NaAc-HAc and 2 M NH_4Ac-HAc buffers. Conditions: As in Fig. 1.

Curve 1: NH_4 buffer, pH 6·5.
Curve 2: NH_4 buffer, pH 5·0.
Curve 3: NH_4 buffer, pH 3·5.
Curve 4: Na buffer, pH 6·5
Curve 5: Na buffer, pH 5·0.
Curve 6: Na buffer, pH 3·5.

It can be seen that the velocity increases for 2 M over 0·2 M buffers are comparatively small at both pH 3·5 and pH 5·0, but that the substrate is much more unstable in 10 M buffers. Furthermore, this decomposition increase is particularly pronounced at pH 5·0. The results suggest that the increases observed are due to the effect of cations. Although Krebs (1942) claimed that monovalent cations do not accelerate the decomposition of O.A.A., his concentrations were so low that the results quoted here do not conflict with his.

Comparison of 2 M Sodium and Ammonium Acetate Buffers.

To investigate further the effect of high concentrations of monovalent cations, the decomposition of O.A.A. was investigated in 2 M NaAc-HAc and 2 M NH_4Ac-HAc buffers at three different H ion concentrations. A typical experiment is shown in Fig. 2.

The results lead to the following conclusions: NH_4 ions are considerably more active than Na ions in accelerating O.A.A. decomposition. The buffer containing the highest concentration of NH_4 ions (viz. pH 6·5) is the most active. Since, in the case of sodium buffer, the reverse is true, it appears as though at a buffer concentration of 2 M the catalysis by hydrogen ions overrides the effect due to sodium ions.

Comparison of the Decomposition in Various Buffers of the Same Molarity.

Differences in the decomposition rate of O.A.A. in various buffers of the same molarity (0·5 M throughout) at the same pH are not very great, but do fall into a definite order.

TABLE 2.

Decomposition of O.A.A. in different 0·5 M buffers.

Conditions: As for Table 1, substituting the appropriate buffers.

Experimental period, 60 mins. for pH 6·0 buffers, 30 mins. pH 3·5 buffers.

Buffer	pH	Total CO_2 outputs of two typical experiments (µl.)			
		1	Theoretical oxalacetate	2	Theoretical oxalacetate
Phosphate	6·0	92	274	101	288
HAc-NaAc	6·0	85	274	88	288
Citrate	6·0	78	274	88	288
Citrate-phosphate	6·0	77	274	83	288
Phthalate	3·5	173	423	111	283
HAc-NaAc	3·5	150	423	99	283
Citrate	3·5	122	423	82	283

As seen from Table 2, the decomposition rates decrease in the order phosphate, acetate, citrate, and citrate-phosphate at pH 6·0, the differences between the last three being very small, and, at pH 3·5, in the order phthalate, acetate, citrate.

Hence, high concentrations of anions may also have some influence on O.A.A. decomposition. On this assumption, the observed O.A.A. decomposition in any one system would be the result of general anionic, cationic and, more specifically, H ion effects.

The Behaviour of O.A.A. in Strongly Acid Solution.

Krebs (1942) reported that at or below pH 1·0, O.A.A. became perfectly stable. This is in accordance with the claim that, in dilute mineral acid, O.A.A. exists almost purely as the keto tautomer, the enol form being that which undergoes spontaneous decarboxylation.

TABLE 3.

Decomposition of O.A.A. at very low pH values.

Conditions: As for Table 2.

Buffer	pH	Experimental period (mins.)	Total CO_2 evolution (μl.)	Theoretical CO_2 (μl.)
HCl-KCl	1·0	30	12	388
HCl-KCl	1·1	15	12	433
HCl-KCl	1·1	60	25	373
HCl-KCl	1·95	30	43	433
HCl-KCl	1·95	60	81	373
10 M HAc-NaAc	2·2	30	53	433
10 M HAc-NaAc	2·2	60	90	373
10 M HAc-NaAc	3·0	30	179	277
10 M HAc-NaAc	3·0	60	299	373
10 M HAc-NaAc	3·5	30	185	277
10 M HAc-NaAc	3·5	60	319	373
10 M HAc-NaAc	4·0	30	203	277
10 M HAc-NaAc	4·0	60	336	373

Table 3 shows results which support those of Krebs, chloride and 10 M acetate buffers having been used. It can be seen that from pH 1·1 to pH 2·2, the decomposition rate is very low, but increases sharply at pH 3·0. Further increases occur as the pH is raised to 3·5 and then to 4·0.

It may be noted that in 2 M acetate buffers the decomposition rate at pH 3·5 exceeds that at pH 4·0, whereas the reverse is true of the 10 M buffers. This must be ascribed to the much higher concentration of either acetate or Na ions, or both, in the more concentrated buffer; in that case, the increase which would be expected due to the increased H ion concentration is masked by the decrease in Na ions.

At pH 1·1, when the autodecomposition rate is almost negligible, the O.A.A. is quantitatively recoverable by aniline citrate. This means that the acid is *not* destroyed by the low pH, but merely converted to a stable (i.e. the keto) form.

II. The Effect of Metals on O.A.A. Decomposition.

The work described in this section shows certain differences in the decomposition of O.A.A. by the two metals investigated, viz. iron and copper. These were in turn investigated under a variety of different experimental conditions.

The Activity of Iron.

The Variation of Fe+++ Activity with Dilution.

These experiments were carried out in 2 M acetate buffers, at different H ion concentrations.

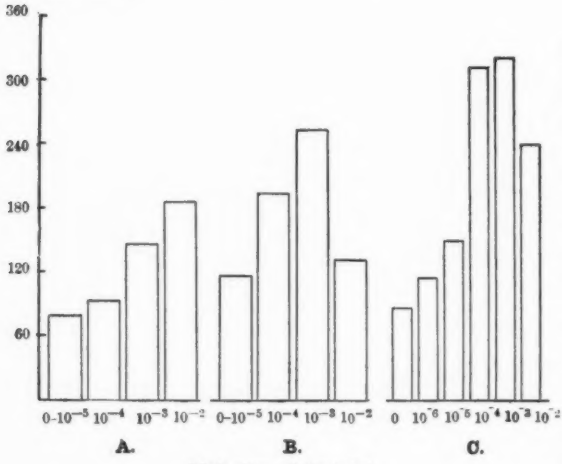

Fig. 3. The variation of Fe+++ catalysis with dilution.

Iron added as $FeCl_3$ to the buffer. 2 M acetate buffers used throughout.

Columns represent total CO_2 evolved for experimental period.

A: pH 6·5, experimental period: 60 minutes.
B: pH 6·0, experimental period: 60 minutes.
C: pH 4·5, experimental period: 15 minutes.

As can be seen from Fig. 3A, the activity at pH 6·5 decreases with dilution, no increases above the autodecomposition rate being observed with Fe+++ concentrations below 10^{-4} M. At and below pH 6·0, however, the effectiveness of 10^{-2} M Fe+++ is severely depressed. This is shown both in Figs. 3B and 3C. At pH 6·0, the 10^{-2} M Fe+++ exhibits a primaerstos, but then the curve flattens out and crosses the 10^{-4} M Fe+++ curve. This has been confirmed in numerous experiments. Also, at pH 4·5, even 10^{-6} M Fe+++ shows slight activity.

Obviously, the metal at a concentration of 10^{-2} M in 2 M acetate buffer at and below pH 6·0 is much less active than *less concentrated* iron, so that these conditions give a quite erroneous picture of its effectiveness.

The Activity of Iron at Varying pH Values.

Again, the medium employed in this series was 2 M acetate buffer, so that the activity changes with varying pH could be compared with those previously described for blood.

For 10^{-2} M Fe+++, an unusual *minimal* activity was observed in the vicinity of pH 6. At this concentration, the following effects may be observed from Fig. 4A. The activities at both pH 5·8 and pH 5·3 lie below that at pH 6·5, although the latter during the last 15 minutes of each experiment shows a flattening out. The activity at pH 5·1 only slightly exceeds that at pH 6·5. From pH 5·1 to pH 4·3, a great increase is observed, and a further, less pronounced, increase occurs to pH 3·7.

On the other hand, Fig. 4B shows the behaviour of 10^{-3} M Fe+++ with pH to be quite regular, the activity increasing further and further as the pH is lowered.

Both 10^{-2} M and 10^{-3} M Fe+++ are still active in surroundings sufficiently acid to stop the autodecomposition of O.A.A. almost completely (Table 4). It is interesting to note that Krebs (1942) reports the total ineffectiveness of Al+++ as a catalyst at or below pH 1·0, whereas Fe+++ is here seen to be very active at pH 1·1.

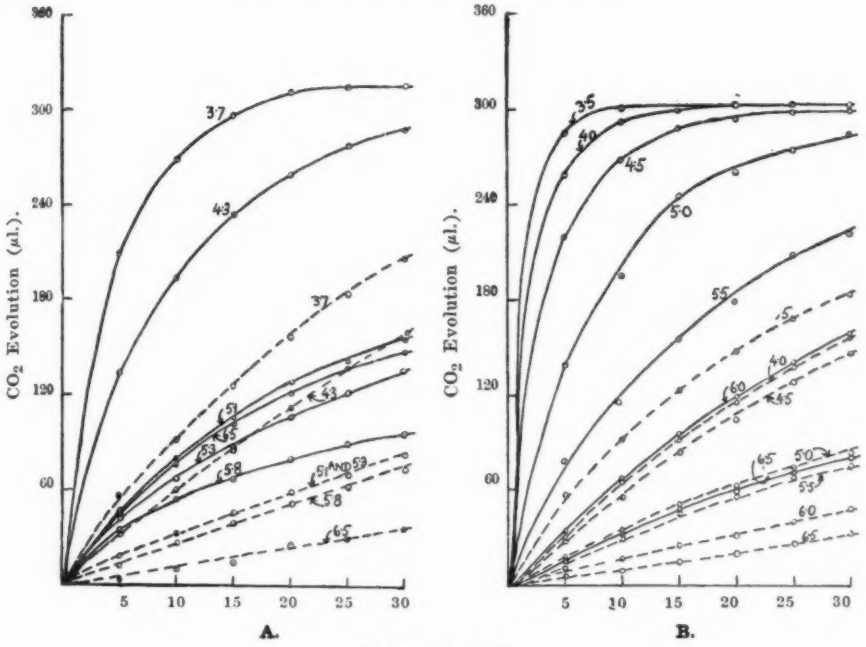

Fig. 4. The variation of iron activity with pH. Conditions: As in Fig. 3.

Dotted curves represent blank decomposition rates and the unbroken curves the catalysed rates. The pH values for all curves are shown in the figures.

A: $Fe^{+++} = 10^{-2}$ M. B: $Fe^{+++} = 10^{-3}$ M.

TABLE 4.

Iron catalysis of O.A.A. decomposition at low H ion concentrations.

Conditions: As in Table 2, but Fe^{+++} added as $FeCl_3$ to the buffer in each cup.

Buffer	pH	Fe^{+++} concentration	Additive CO_2 evolutions (μl.) over five-minutely intervals after tipping in the substrate						Total blank CO_2 (buffer only) (μl.)
			1	2	3	4	5	6	
KCl-HCl	1·1	10^{-2}	343	393	401	fin.*	—	—	11
KCl-HCl	1·9	10^{-2}	366	409	413	fin.*	—	—	26
10 M HAc-NaAc	2·2	10^{-2}	160	210	223	231	234	fin.	34
10 M HAc-NaAc	2·2	10^{-3}	133	195	216	231	235	fin.	34
10 M HAc-NaAc	3·0	10^{-2}	228	341	fin.	—	—	—	84
10 M HAc-NaAc	3·0	10^{-3}	218	239	fin.	—	—	—	84
10 M HAc-NaAc	3·5	10^{-2}	241	244	fin.	—	—	—	92
10 M HAc-NaAc	3·5	10^{-3}	236	243	fin.	—	—	—	92
10 M HAc-NaAc	4·0	10^{-2}	248	252	fin.	—	—	—	99
10 M HAc-NaAc	4·0	10^{-3}	242	255	fin.	—	—	—	99

* fin. signifies cessation of CO_2 evolution.

Both this and the preceding section indicate that at about pH 6·0, 10^{-2} M Fe+++ in 2 M acetate buffer exhibits a curious weakness in its effect on O.A.A. decomposition. This anomalous behaviour is not found in the case of blood, and suggests that metals may differ in their mode of action from the biological systems.

The Variation of Fe+++ Activity with Buffer Concentration.

It was shown earlier that the decomposition rate of O.A.A. increased quite considerably with increasing buffer concentration. The effect of 10^{-2} M and 10^{-3} M Fe+++ at the three buffer molarities (10 M, 2 M and 0·2 M) was also investigated, both at pH 5·0 and pH 3·5. Decomposition rates are shown in Fig. 5 for pH 5·0.

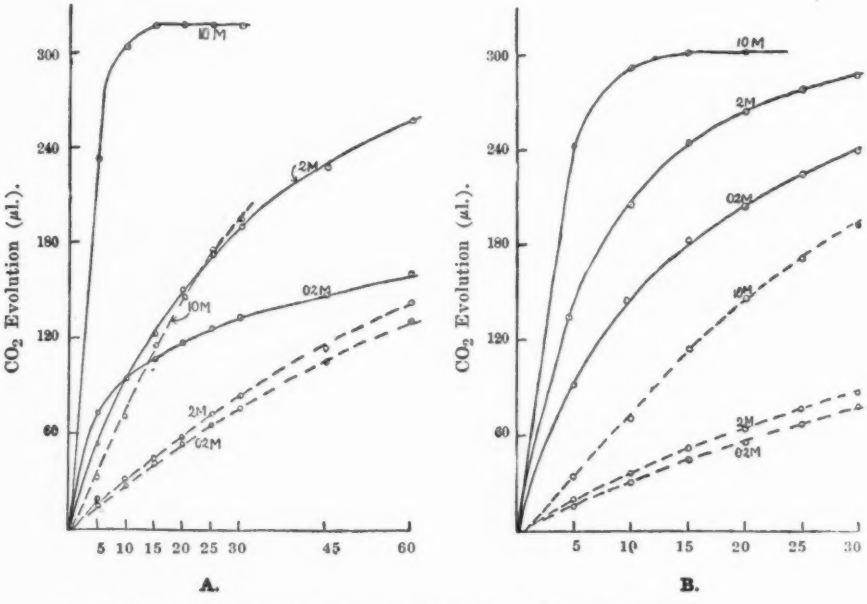

Fig. 5. The effect of buffer concentration on iron activity at pH 5·0.

Conditions: As in Fig. 3, substituting the appropriate buffer. The broken curves are blank decomposition rates, the unbroken curves rates with catalyst. The molarities of the acetate buffers are shown in the figures.

A: 10^{-2} M Fe+++. B: 10^{-3} M Fe+++.

In all cases, 10^{-3} M Fe+++ is more active than 10^{-2} M Fe+++. At both concentrations of Fe+++, the activity is maximal in the strongest buffer. The differences between the catalysed decomposition rates in 2 M and 0·2 M buffers are much more pronounced than between the corresponding blank decomposition rates. Again, 10^{-2} M Fe+++ departs from the otherwise regular behaviour of these experiments, in that it shows a more rapid initial activity in 0·2 M buffer than in 2 M buffer, but the curve for the former flattens out noticeably after the first five minutes.

At pH 3·5, the decomposition is so rapid that it need not be shown graphically. The differences of effectiveness in the various buffers here are slight, but again Fe+++ is most active in the strongest buffer.

These findings would indicate that the catalysis is not simply one due to Fe+++ ions alone, but involves the buffer as well. It may be that the catalyst is a ferric acetate complex, or else that Fe+++ catalyses the decomposition of some type of substrate-buffer complex, the catalysis not being due to a simple O.A.A.-ferric complex (as with Al+++).

Activity of Fe+++ in Other Buffers.

From Table 5 it can be seen that buffer composition has a very pronounced effect on the activity of iron. The most puzzling results are those observed in citrate-phosphate buffer. Although in citrate alone, and in phosphate alone, the catalytic effect is low, in the mixed buffer it is definitely greater even than in acetate buffer. At pH 6·0, then, the effectiveness of Fe+++ decreases in the order: citrate-phosphate, acetate, phosphate and citrate for these four buffers.

TABLE 5.

The catalytic effect of iron in various buffers.

Conditions: As for Table 4. All buffers 0·5 M.

Experimental period: Indicated by the figures in brackets after the CO_2 evolution value, in minutes.

Buffer	pH	Molarity of Fe+++	Experimental CO_2 evolution (μl.)	Corresponding blank CO_2 evolution (μl.)
Citrate	6·0	10^{-2}	111 (60)	88
Citrate	6·0	10^{-3}	96 (60)	88
Phosphate	6·0	10^{-2}	172 (60)	101
Phosphate	6·0	10^{-3}	109 (60)	101
Acetate	6·0	10^{-2}	230 (60)	88
Acetate	6·0	10^{-3}	186 (60)	88
Citrate-phosphate	6·0	10^{-2}	311 (45)	70
Citrate-phosphate	6·0	10^{-3}	171 (60)	89
Citrate	3·5	10^{-2}	267 (60)	111
Citrate	3·5	10^{-3}	160 (60)	111
Acetate	3·5	10^{-2}	375 (15)	—
Acetate	3·5	10^{-3}	393 (15)	—
Phthalate	3·5	10^{-2}	379 (15)	—
Phthalate	3·5	10^{-3}	394 (15)	—

At pH 3·5, the behaviour is more regular: Both concentrations of Fe+++ are least active in the citrate buffer, whereas in phthalate and acetate the activities are almost equal, that in phthalate usually slightly exceeding that in acetate.

These results confirm the earlier suggestion, viz. that the effectiveness of iron is in some way intimately connected with the buffers employed. (This is not simply a solubility effect, since some of the above systems, in spite of showing precipitation of ferric compounds, still proved very active.)

The Activity of Copper.

Since iron showed several idiosyncrasies in its behaviour as a catalyst in the decomposition of O.A.A., another active metal was investigated under the same sets of experimental condi-

tions. Copper was chosen because it does not form such involved complexes with acetate as iron.

The variation of activity of copper (Cu++) with dilution at pH 6·5 has been reported previously (Nossal, 1948). It was found that copper was more active than iron at equivalent concentrations. However, between 10^{-4} M and 10^{-5} M Cu++, a sharp drop in activity occurs, and the effect at 10^{-6} M Cu++ is almost negligible.

The Activity of Copper at Varying pH Values.

Changes in the activity of copper with pH were examined at metal concentrations of 10^{-3} M and 10^{-4} M. Representative results are shown in Fig. 6.

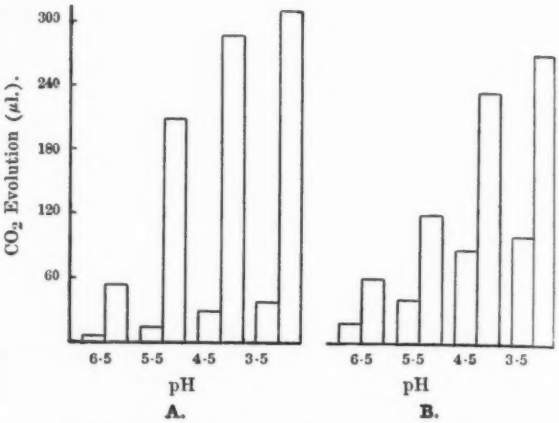

Fig. 6. Variation of copper activity with pH.

Conditions: As for Fig. 4.

The pairs of columns represent total CO_2 evolved at the pH indicated over the respective experimental periods, the smaller column on the left being CO_2 due to autodecomposition, and that on the right being the catalysed rate.

A: 10^{-3} M Cu++, experimental period: 5 minutes.
B: 10^{-4} M Cu++, experimental period: 15 minutes.

Unlike the case with iron, progressive increases in activity occur when the pH is lowered from 6·5 to 3·5. The higher Cu++ concentration shows greater effectiveness at all four H ion concentrations. At 10^{-3} M Cu++ the rates below pH 6·5 are so rapid that they appear very nearly equal at pH 5·5, 4·5 and 3·5 (contrast with Fig. 4). There is one anomaly, viz. at pH 6·5 and 10^{-3} M Cu++, the CO_2 evolution definitely levels off at ca. 16 p.c. below the theoretical value after 45 minutes.

The Variation of Cu++ Activity with Buffer Concentration.

While iron was most effective in the strongest acetate buffer, Fig. 7 shows that the reverse is true for copper. At both pH values tested, the most active copper catalysis occurs in the least concentrated buffer.

Unlike Fe+++, copper does not form tetravalent co-ordinated acetate complexes. Since the catalytic effect of Cu++ is minimal in the most concentrated buffer, it appears as though the free cupric ions constitute the actual catalyst; by a mass action effect, formation of the highly unstable O.A.A.-copper complex will be least in the strongest buffer, owing to increased O.A.A.-buffer complex formation.

The Activity of Copper in Other Buffers.

Table 6 shows the behaviour of 10^{-2} M to 10^{-4} M Cu++ in the four pH 6·0 buffers used previously. The following differences from the observed iron effects are seen.

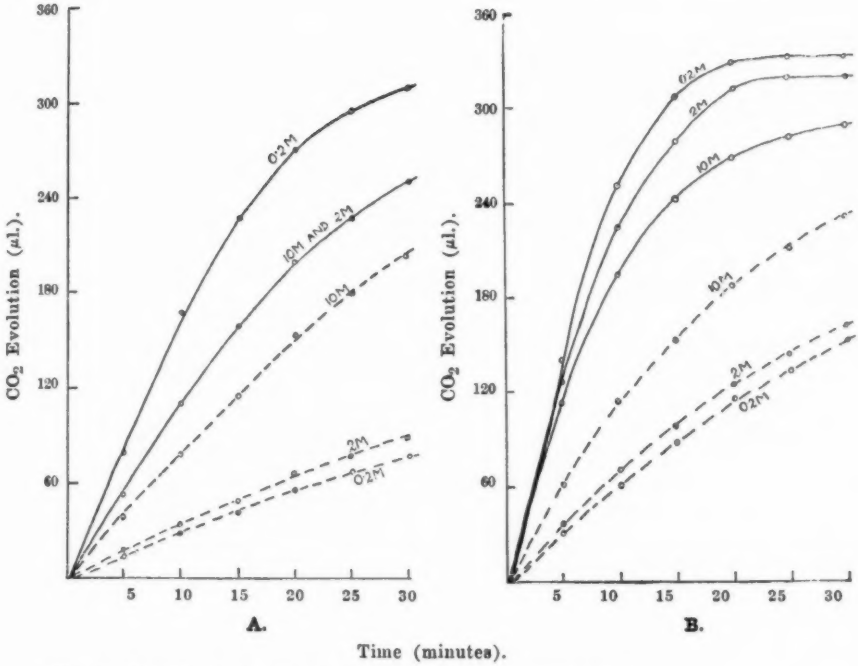

Fig. 7. Variation of copper activity with buffer concentration.
Conditions: As in Fig. 5.
Cu++ = 10–4 M. A: pH 5·0. B: pH 3·5.

TABLE 6.

The activity of copper in various pH 6·0 buffers.

Conditions: As in Table 5, except that buffer molarity was 0·2 throughout.

Buffer	Molarity of copper	Experimental CO_2 evolution (duration in minutes indicated by the figures in brackets) µl.	Corresponding blank CO_2 µl.	Theoretical CO_2 µl.
Citrate	10–2	307 (5)	15	356
Citrate	10–3	166 (30)	59	356
Citrate	10–4	89 (30)	52	350
Citrate-phosphate	10–2	337 (5)	17	356
Citrate-phosphate	10–3	278 (30)	64	356
Citrate-phosphate	10–4	116 (30)	54	350
Phosphate	10–2	352 (5)	20	356
Phosphate	10–3	324 (30)	77	356
Phosphate	10–4	300 (30)	81	350
Acetate	10–2	71 (5)	17	356
Acetate	10–3	235 (5)	17	356
Acetate	10–3	334 (30)	68	356
Acetate	10–4	349 (30)	64	350

For 10^{-3} and 10^{-4} M Cu++, increasing activity is found for the following order of buffers: citrate, citrate-phosphate, phosphate and acetate. The same effect is observed for 10^{-2} M Cu++ for the first three, but in acetate a severe depression occurs, so that 10^{-3} M Cu++ is much more active than 10^{-2} M Cu++. Thus *both* iron *and* copper in high concentrations show abnormally low activities in acetate buffers at pH 6·0.

In the pH 3·5 buffers, 10^{-3} M Cu++ causes quantitative decomposition in the acetate and phthalate buffers in 10–15 minutes, the effect in acetate being somewhat more rapid than in phthalate. In citrate, catalysis is still rapid, but definitely less so than in the above. The differences for the three buffers are shown better by 10^{-4} M Cu++, when the effectiveness decreases in the order: acetate, phthalate, citrate, with the last lagging well behind the other two.

DISCUSSION.

Although previous experiments had indicated (Nossal, 1948) that the decarboxylation of O.A.A. in blood was *not* contributed to by iron and copper ions, it appeared likely that at least part of the activity of systems derived from blood must be ascribed to non-enzymatic catalysts. It seemed worth while, therefore, to make a detailed study of simple inorganic and physical factors. It is not suggested that the results necessarily have a direct bearing on the decarboxylation of O.A.A. *in vivo*, but certain of the conclusions reached can be applied to the investigation of the known decarboxylases.

The abrupt stabilization of O.A.A. somewhere between pH 3·0 and pH 2·2 seems due to a shift of the keto-enol equilibrium. Although in water O.A.A. has been claimed to exist largely in the keto form, there must still be an equilibrium in which a certain amount of enol form occurs, the latter accounting for the observed autodecomposition rates above pH 2·2. Since Fe^{+++} is found to be very active even at pH 1·1, some enol form may be present even at that pH, but the amount must be so small that the autodecomposition rate is almost negligible.

The pronounced acceleration observed in high molarity buffers could be ascribed to formation of trisodium salt which, owing to its more rapid decomposition, would cause a continuous shift of the equilibrium towards enol formation. This suggestion is supported by the finding that increases in decomposition rate in 10 M buffers are more pronounced at pH 5·0 (where the buffer contains higher concentrations of Na^+ or NH_4^+) than at pH 3·5. Comparison of NH_4Ac and NaAc buffers of the same molarity and H ion concentration shows NH_4^+ to be much more active than Na^+.

Several differences are observed when the effectiveness of iron and copper is compared under varying experimental conditions.

The abnormally low activity of iron under certain conditions (10^{-2} M Fe^{+++}, 2 M acetate buffer, pH $<6{\cdot}5$) and of copper (10^{-2} M Cu++, 2 M or 0·2 M acetate buffer, pH 6·0) cannot be explained simply. It is also difficult to see why Fe^{+++} should be much more active in phosphate-citrate buffer than in either phosphate or citrate alone.

It appears likely that O.A.A. decomposition rate usually represents the sum of the rates of a number of complexes of differing stabilities. In a buffer alone, these complexes may involve any of the cations and anions present (or the undissociated acid). This would account for the differences observed in various buffers of the same molarity and H ion concentration.

Maximum complex formation, according to Law of Mass Action, tends to occur with the constituents present in the highest concentrations. In the presence of strong buffers, for instance, formation of the more unstable metal-O.A.A. complexes may be suppressed, the effectiveness of the metal thus appearing deceptively low.

Copper, being least active in the most concentrated buffer, may act as free Cu^{++} ions; in the case of iron, on the other hand, maximum effectiveness was found in the strongest buffer, so that the catalyst may actually be a tetravalent co-ordination compound of iron and acetate.

Thus, the non-enzymatic decarboxylation of O.A.A. is seen to be complex and the interpretation of results obtained in the investigation of the enzymatic decomposition at non-physiological pH, in artificial buffers, etc. must be made with caution.

Results of such an investigation at any one H ion concentration and in only one molarity of one particular buffer can obviously lead to an erroneous conception of the enzymatic mechanism.

The methods used in this work have been applied to the decomposition of O.A.A. in blood, and the results of that investigation will appear shortly.

SUMMARY.

The decomposition of oxalacetic acid (O.A.A.) has been studied in detail under varying experimental conditions, including the presence of ferric and cupric ions.

Solutions of O.A.A., if left unneutralized have been shown to be much more stable than neutral samples.

The influences of temperature, buffer concentration, buffer composition and high hydrogen ion concentration are described.

The activity of both metals increases greatly from pH 6·5 to pH 3·5.

Maximal effectiveness of Fe^{+++} in acetate buffer at any one pH was found in the concentrated buffer.

The effect of copper differs in several respects from that of iron. It shows a more regular behaviour towards dilution and pH. Furthermore, copper is found to be least active in the most concentrated acetate buffer.

The results are discussed in the light of competitive complex formation, and attention is drawn to the bearing of this investigation on the study of enzymatic decarboxylation of O.A.A.

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THE INFLUENCE OF MINERAL CONTENT ON THE LOSS OF VITAMIN B_1 IN BAKING BREAD

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In the course of a survey of the thermal destruction of vitamin B_1 in cooking or processing foodstuffs, some attention was paid to published work concerned with the loss of this vitamin during the baking of bread. Most of the data available clearly show the influence of the two well-recognized factors, time and temperature, on the loss of vitamin B_1 (as do the figures of Down and Meckel, 1943, which show increasing losses with increasing toasting time), and several authors underline this by reference to the temperature gradient in a baking loaf and to the high losses of vitamin in the crust and the corresponding low losses in the crumb.

The average overall loss to be expected in baking a loaf would appear to be of the order of rather less than 20 p.c. whatever the actual conditions used. However, Dawson and Martin (1941 and 1942) have shown that, in baking, high extraction flours show greater losses of vitamin B_1 than normal white flours, and Schultz, Atkin and Frey (1942) record a rather higher loss from whole meal than from white bread.

Golberg and Thorp (1946) also found greater destruction in baking doughs from high extraction flours, but attributed it to the longer baking time required. This explanation will not hold, however, as Dawson and Martin's experiments were carried out under constant conditions.

The latter authors suggested two possible explanations for these different rates of destruction (i) changes in pH, (ii) absorption of free aneurin from the dough by the yeast during fermentation and protection of it in the subsequent baking. This phenomenon being more complete in the lower extraction flours of lower vitamin B_1 content would give rise to higher rates of destruction in the higher extraction flours if the vitamin inside the yeast cell was less vulnerable to heat, and especially if the vitamin B_1 in these flours is bound to the bran and is less available to the yeast.

In fact, they could find little difference in the pH values of the different breads (the doughs were not tested), but they did claim that vitamin B_1 added as high vitamin yeast is much more stable than that added as synthetic aneurin. Unfortunately, Petit, Guillot and Guillemet (1945) suggest the reverse, and Schultz, Atkin and Frey (1942) could find no difference. The

greater part of the B_1 in yeast is in the form of cocarboxylase and Lincoln *et al.* (1944) working with cereals, Myrback and Vallin (1944) with meat, and Farrer (1945b) with buffer solutions all agree that cocarboxylase is less stable to heat than aneurin. There would thus appear to be a balance between the greater vulnerability of cocarboxylase and the protective effect of a slightly lower pH inside the yeast cell than in the surrounding dough. Finally, there is good evidence that bound aneurin does not occur in cereals (see Discussion).

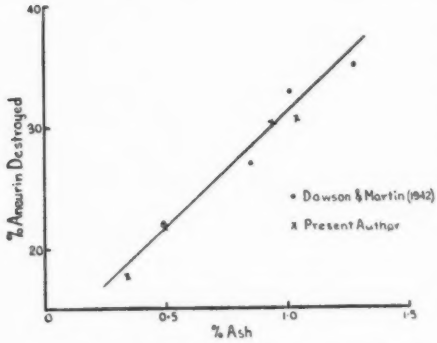

Fig. 1. Relationship between Aneurin destroyed in baking bread and mineral content of original flour.

On closely studying Dawson and Martin's results, the present author noticed that if one plots percentage ash in the flours used by these authors against the percentage destruction of vitamin B_1 found, one obtains the linear relationship shown in Fig. 1. As the concentration of buffer salts (Farrer, 1947) and of yeast extract (Farrer, unpublished work) have already been shown to be capable of modifying aneurin destruction, this relationship was not particularly surprising. It was decided to confirm it with Australian flours.

EXPERIMENTAL.

Four samples of flour of 74·9 p.c. (control), 85 p.c., 98 p.c. and 100 p.c. extraction were kindly made available by Mr. W. L. Jones of W. S. Kimpton & Sons, Kensington, who also baked the bread. The doughs were prepared according to standard practice and the loaves were baked in an electrically heated, experimental rotary oven for 30 minutes at 475° F. (248° C.). The following determinations were made on flours, doughs and breads (crumb only).

Ash (on flour). The total mineral content was determined by conventional methods.

pH (on dough and bread). The pH values of the doughs were determined directly with the glass electrode; confirmatory tests were done with the quinhydrone electrode. There was good agreement in each case. Similar determinations were made on the bread samples, moistening each sample with distilled water.

Moisture. Samples of flour and air-dried dough and bread (q.v.) were dried to constant weight at 102 ± 2° C.

Vitamin B_1. Samples of dough and bread were air-dried at 100° F. and stored in air-tight jars. Total vitamin B_1 was determined as follows:

0·5–2·0 gm. of sample were weighed out and heated with about 15 ml. of N/10 H_2SO_4 on a water-bath for 10 minutes with continual stirring. 5 ml. of acetate buffer, pH 4·5, were added and the mixture cooled: 80–100 mg. each of taka-diastase and papain were added and the mixture incubated overnight at 37° C. After centrifuging, the supernatant liquor was made up to 25 ml. and 10 ml. were washed 3 times with water-saturated butyl alcohol, after the addition of 2 ml. of conc. HCl. The final aqueous layer was then made up to 20

ml. and assayed in triplicate by the usual thiochrome method using n-butyl alcohol. The thiochrome fluorescence was read in a Coleman Model 12A Photofluorometer. Free vitamin B_1 (aneurin) was determined on the flour samples by omitting the enzyme digestion and proceeding with the assay on the acid extracts.

Booth (1940) has commented that "wheat contains little or no protein-bound vitamin B_1". To check this an attempt to determine protein-bound vitamin B_1 (by difference) in the 98 p.c. extraction flour was made by extracting the sample with trichloracetic acid and determining total vitamin B_1 on the extract. However, the value obtained was exactly the same as that obtained by H_2SO_4 extraction.

RESULTS.

The moisture values were used for calculating all vitamin figures on a dry basis and have not been recorded individually. The pH values are recorded in Table 1 and the vitamin and ash values in Tables 2, 3 and 4. The relationship between vitamin destruction and mineral content is shown in Fig. 1.

TABLE 1.

pH of dough and breads (glass electrode) prepared from different extraction flours.

P.c. extraction	pH of dough	pH of flour
74·9	5·68	5·22
85·0	5·62	5·43
98·0	5·84	5·70
100·0	5·98	5·76

TABLE 2.

Free and phosphorylated vitamin B_1 in flours.

P.c. extraction	Free aneurin µg./g.	Total vitamin B_1 µg./g.	P.c. as cocarboxylase
74·9	1·24	1·35	8·2
85·0	3·04	3·49	12·9
98·0	4·4	4·6	11·3
100·0	4·84	5·30	9·0

TABLE 3.

Ash in flour samples.

P.c. extraction	P.c. ash
74·9	0·35
85·0	0·49
98·0	0·97
100·0	1·06

TABLE 4.

Vitamin B_1 (total) in dough and bread.

Flour used p.c. extraction	μg. B_1/gm. dough (dry)	μg. B_1/gm. bread (dry)	P.c. loss
74·9	1·30	1·07	18·0
85·0	3·40	2·66	21·7
98·0	4·65	3·24	30·0
100·0	5·30	3·66	31·0

DISCUSSION.

It is seen that these results show the same linear relation between p.c. ash in the flour and p.c. vitamin B_1 destroyed as those of Dawson and Martin. That they fit the same curve may be fortuitous as the baking time and temperature are less than those used by Dawson and Martin. There is little doubt that the actual oven temperature is not related to the temperature reached by the crumb (on which all assays were done) which rarely, if ever, rises higher than 100° C. The difference in time is more significant.

The pH of both dough and bread shows a significant rise with increasing percentage extraction of the flour. This at first sight may seem sufficient to account for the parallel increasing destruction of aneurin. However, in view of the very slight increase in k (the velocity coefficient for aneurin destruction) between pH 5 and 6 found in phosphate buffer solutions (Farrer, 1945a) (and phosphate seems to be the predominating anion in cereals, as, indeed, in most foods), it seems likely that this increase in destruction is not solely (if at all) a pH effect but may also be related to the increase in inorganic constituents. This may be hard to conceive, for, even in white flours the minerals present are very greatly in excess of the vitamin B_1 but, although the latter increases at a greater rate than the mineral content as the percentage extraction of the flour rises, the increase in the mineral concentration in the aqueous phase is likely to play a much more important part in influencing vitamin B_1 destruction than the increase in concentration of the vitamin B_1 in the aqueous phase.

The only other explanation of the phenomenon discussed would appear to require an increase in the amount of more vulnerable cocarboxylase in the higher extraction flours. This would seem to be discounted by Booth (1940) as already mentioned and by other workers since who agree on the virtual absence of cocarboxylase (Shetlar and Lyman, 1941; Obermeyer *et al.*, 1944; and Teysseire, 1945). The results in Table 2 for the flours studied in this work confirm that cocarboxylase forms only a small part of the vitamin B_1 present and, moreover, does not increase as the degree of extraction is increased.

SUMMARY.

It was noticed that in the results of Dawson and Martin (1941 and 1942) there was a linear relation between the percentage destruction of vitamin B_1 in baking bread and the percentage of ash in the flours used. Baking losses increased as the mineral content rose.

Experiments were carried out on Australian flours of four different degrees of extraction (and, hence, of mineral content) and a similar linear relationship was obtained.

This increase in destruction cannot be attributed to increasing pH or cocarboxylase content and appears to be related to an increased concentration of electrolytes in the aqueous phase as has already been observed in experiments with buffer solutions.

Acknowledgments. The author is particularly indebted to Mr. W. L. Jones of W. S Kimpton & Sons, Kensington, for providing the flour samples and baking the loaves. Thanks are due to Miss P. G. Morrison for assistance with the analytical work and to the Directors of the Kraft Walker Cheese Co. Pty. Ltd. in whose laboratories the work was done, for permission to publish the results.

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SALMONELLA INFECTIONS IN QUEENSLAND

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In a paper shortly to be published, Miss Atkinson and her colleagues will be recording a large number of Salmonella infections from Queensland, the great majority having been isolated during a severe epidemic of infantile gastro-enteritis in Brisbane in the latter half of 1947. The purpose of the present communication is to describe the circumstances in which most of the strains were obtained. The state of our published knowledge when the investigation commenced is shown in Table 1, together with the earlier records of Mr. G. C. Simmons, who had already for some time been studying Salmonella infections

TABLE 1.

Species of Salmonella previously known from Queensland.

Group	Species	Locality	Source	Author
B	*typhi-murium*	N.Q.	Adults with gastro-enteritis and Salmonella fever	Atkinson *et al.*, 1947
			Epidemic in guinea-pigs	Atkinson *et al.*, 1944, 1947
		Brisbane	Children with meningitis	Atkinson *et al.*, 1947
			Flummery	Atkinson *et al.*, 1947
C	*choleraesuis* var. *kunzendorf*	S.Q.	Pigs	Simmons (personal communication)
	oslo	N.Q.	Adults with gastro-enteritis	Atkinson *et al.*, 1947
	muenchen	S.Q.	Chickens	Simmons (personal communication)
	bovis-morbificans	Brisbane	Child with gastro-enteritis (isolated, April, 1945)	Atkinson *et al.*, 1947
		N.Q.	Adults with gastro-enteritis	Atkinson *et al.*, 1947
D*	*pullorum*	S.Q.	Chickens	Simmons (personal communication)
E	*newington*	N.Q.	Adults with gastro-enteritis	Atkinson *et al.*, 1947
	newbrunswick	N.Q.	Adults with food-poisoning and gastro-enteritis	Atkinson *et al.*, 1947
—	*mississippi*	N.Q.	Adults with gastro-enteritis	Atkinson *et al.*, 1947
	atherton	N.Q.	Adults with gastro-enteritis	Ferris *et al.*, 1945 Atkinson *et al.*, 1947

* *S. typhi* omitted.

The methods used in the investigation have been described in another paper (Mackerras and Mackerras, 1949).

in animals at the Queensland Department of Agriculture's Animal Health Station, Yeerongpilly, and whose experience was available to us from the beginning. The North Queensland records shown were all from Army hospitals during the war, and the strains from children were isolated by the Brisbane General Hospital laboratory.

MATERIALS.

The material examined from July, 1947, to May, 1948, is set out in Table 2, the greater part having been collected before the end of the year.

TABLE 2.

Material examined during present investigation.

Material	Group	No. of individuals	No. of specimens	Salmonellas isolated
Faeces	Survey: children < 1 year old	124	158	9
	children 1–2 year old	37	51	4
	children > 2 year old	111	119	5
	adults	295	333	9
	Total survey material	567	661	27
	Patients with gastro-enteritis	217	845	177
Post-mortems	Children	52*	243	34*
Human	Total	800*	1,749	211*
Animal	Rats, mice, cats, cockroaches	558	986	15
	Totals:	1,358	2,735	226

* In the post-mortem series, 36 individuals and 27 strains of Salmonella are included also in the gastro-enteritis group examined during life, and therefore do not contribute to the totals.

The survey comprised: infants and young children in hospital but not diagnosed as gastro-enteritis; infants from infant welfare centres; infants, children, and adult staff from an institution for homeless children; infants, children and adults, who were relatives or contacts of known cases of Salmonella infection; and mothers and nursing staff in a maternity hospital. The gastro-enteritis group was composed almost entirely of infants under 2 years of age.

SALMONELLA INFECTIONS.

The species of Salmonella encountered by us, or received from other laboratories, are listed in Table 3, isolation of a species from a single individual being treated as one infection, irrespective of how many times it was isolated or from how many parts of the body. Super-added infections are those in which one Salmonella (or sometimes a dysentery bacillus) was present in earlier examinations and a different Salmonella was found later. We have, further, listed all

clinically active salmonellosis as gastro-enteritis, for that group included the vast majority of the cases. Two were typical examples of food poisoning, in that they were short, sharp, and associated with consumption of a particular dish, but the distinction is one of degree rather than of kind. There were no cases of "primary" salmonella septicaemia, though septicaemic manifestations were not infrequent at some stage of the gastro-enteritis in the infants. Salmonella fever was not seen.

TABLE 3.

Species of Salmonella isolated in present investigation.

Group	Species	Gastro-enteritis	Super-added infec-tions	Carriers	Animals	Total
B	*S. typhi-murium*	23	1	1	brown rat 1; cockroach 1	27
	S. derby	3	—	1	—	4
	S. chester	2	—	1	brown rat 1	4
C	*S. paratyphi C*	—	—	1	brown rat 1	2
	S. bovis-morbificans	136	9	10	cat 1; brown rat 1; mice 3; cockroaches 3	163
	S. kottbus	—	—	1	—	1
	S. newport	1	—	—	—	1
	S. muenchen	1	—	2	—	3
D	*S. pullorum*	—	—	1	—	1
E	*S. newington*	—	1	1	—	2
	S. meleagridis	—	—	—	brown rat 1	1
	S. amager	1	—	—	—	1
—	*S. adelaide*	7	2	5	brown rat 1	15
	S. sp.	—	—	—	mouse 1	1

In the following notes it will be convenient to consider the species in the order of their frequency rather than in the systematic sequence adopted in the Table.

S. bovis-morbificans.

This species was responsible for a major epidemic of gastro-enteritis, which was restricted to the Brisbane area and almost exclusively to young children. Its chief manifestations were severe vomiting, moderate to severe diarrhoea, extreme dehydration, a marked tendency to improve and relapse, and an over-all mortality of 37 p.c., which reached a sectional maximum of 60 p.c. in the 9–12-months-old group. Ninety-one p.c. of cases were under 2 years old, the youngest patient being 2 days and the oldest 57 years. The duration of illness varied from a few days to many weeks in those that survived, and averaged 26 days in those that died.

Post-mortem, there was little more in the small or large intestine than patchy engorge-

ment and increased cellular content of the mucosa, some engorgement of mesenteric vessels, and usually swelling of associated lymph nodes and glands, with widening of the sinuses and moderate macrophage development. The outstanding feature was in the liver, which had undergone almost complete fatty transformation, with compression of sinusoids, loss of architecture, and few unaltered parenchymal cells, so that one almost needed the portal tracts to recognize the tissue as liver and not as a closely packed mass of peculiar fat cells. Tidswell (in Harper, 1932) reports exactly similar findings in the 1930–31 epidemic in Sydney.

We generally received four tissues for culture from the hospital, the results from proven cases being:

Spleen: 34 examined, 21 positive.	Small intestine: 34 examined, 23 positive.
Liver: 32 examined, 22 positive.	Colon: 33 examined, 22 positive.

In only 13 instances were the organisms isolated from all four situations in the same patient.

In addition to these (and the faeces in life), *S. bovis-morbificans* was isolated occasionally, either by the General Hospital Laboratory or ourselves, from: blood (3), urine (1), mesenteric gland (1), lung (1), middle ear (4), meninges (1), cerebrospinal fluid (1).

The infection was very much milder in older children and adults, and these provided nearly all the symptomless carriers recorded in Table 3. The severity of clinical manifestations also fell rapidly with the decline in the epidemic, there being only one death in 25 bacteriologically proven cases with onset since 1st November. Normally, it would appear, *S. bovis-morbificans* infections are no more severe than those caused by other members of the genus.

The clinical features and treatment of the infection are being described separately by Drs. Stable and Philpott (1948), and its epidemiology and prevention by ourselves (in preparation). We may, however, give brief epidemiological notes for comparison with the other species. We know nothing of its origin, but during the developed phase the infection was predominantly (62 p.c.) institutional and its spread was essentially contaminative from the infected children. The risk was increased by the facts that older children often had inapparent infections, and infectivity of the active cases continued, sometimes for long periods, after clinical recovery. Thus, in 51 cases followed, the duration of infectivity was: less than 4 weeks in 18, 4–12 weeks in 25, more than 12 weeks in 8. The longest infection encountered was a baby with onset 2 days after birth and still a carrier 7 months later. Three others remained infective for more than 4 months. As regards the neo-natal cases, infection was doubtless brought into the hospital by women who were unconscious carriers, though only one *bovis-morbificans* infection was found in 113 mothers examined (but see *muenchen* below). A cat suffering from acute enteritis, rodents and cockroaches were found infected, but appear to have been minor factors in spreading the infection, and there was no indication of a major animal reservoir from which infection in the human population was continually being replenished. Mr. Simmons has isolated *S. bovis-morbificans* from a duck in Brisbane.

S. typhi-murium.

The *typhi-murium* infections were quite severe, and there were three deaths, one of which however, was complicated by a super-added *bovis-morbificans* infection. This was a girl aged six months who had a fairly severe attack of gastro-enteritis, with *S. typhi-murium* in the stools. When apparently convalescent, she relapsed and died, and *typhi-murium* was isolated from the spleen and liver, but *bovis-morbificans* alone from the intestines.

Neither in age distribution nor clinical features could these infections be distinguished

from the epidemic series. The mean duration of infectivity (3 weeks) was, however, less, and the maximum (9 weeks) decidedly less. In addition, there were two important epidemiological differences: (1) The infections were rather evenly scattered in time, with a slight peak in September-October, the monthly distribution of clinically active cases from July to May being: 1, 1, 5, 6, 2, 1, 3, 2, 1, 0, 1. (2) They were widely scattered in space, occurring at random all over Brisbane, with few institutional infections, which were the only indication of any relationship between cases.

The only healthy carrier encountered was a nurse in a maternity hospital; she had no detectable relation, either as receiver or donor, to any of the other infections. As with all other adult Salmonella carriers we have examined, her infection appears to have been transitory, for she quickly became negative on re-examination. *S. typhi-murium* is widely distributed in animals, including rodents, and a rodent reservoir would fit our epidemiological picture very well, as Draper (1944, 1945) suggested for a similar series in Adelaide. However, we found this organism only once in 241 rats and 106 mice examined. Mr. Simmons has isolated it from pigs at Brisbane and calves at Warwick.

S. adelaide.

This series was particularly interesting, because we know of only two previous infections with this species in Australia (Atkinson, 1943; Atkinson and Woodroofe, 1944; Atkinson, Woodroofe and Macbeth, 1944), and four in Great Britain (Taylor, 1947).[1] *S. adelaide* appears to be of relatively low pathogenicity, for the clinical infections, though sharp, were not severe, and the proportion of symptomless carriers was high. The age incidence of clinical infections was not unusual (5 under 1 year, one 2 years old, and 1 adult), the duration of infectivity from 2 to 9 weeks, there were no complications, and no deaths. The two presumably intercurrent infections were found at routine examinations of convalescents (one from bacillary dysentery, one from a *bovis-morbificans* infection), and neither was associated with any clinical indication of its presence.

The carriers comprised two babies in an institution for homeless children and a family in a temporary housing camp. The former were found at a survey, were three and six months old respectively, had no more history of "gastric upsets" than any of the other infants, and no known relation to any other cases. One lost his infection between the 16th and 30th day after it was discovered;[2] the other left the institution before he could be examined further. The family was interesting. One twin, aged one month, had died with a *bovis-morbificans* infection, the other had gastro-enteritis at the same time but remained at home. Six weeks later the whole family (mother, girl aged 5, boy aged 15 months, and the remaining twin) were surveyed; the twin had an *S. adelaide* infection, the others were negative. Three weeks after that, the mother, the girl, and the twin had *S. adelaide* in their faeces; they were quite well. Unfortunately we were not able to follow them further.

The evidence from carriers and the finding of infections in hospital suggest that *S. adelaide* can be spread contaminatively in the same way as *S. bovis-morbificans*. The other cases, however, were as widely scattered as the *typhi-murium* infections, and with as little association between them; the discovery of *S. adelaide* in a suburban rat may point to a common basic epidemiology.

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[1] Miss Atkinson (personal communication) has identified four more infections with *S. adelaide*, three from children with gastro-enteritis, of whom one died, and one from a soldier at Morotoi.

[2] These children were examined twice in the survey, and those found to harbour Salmonella were re-examined at intervals until they gave three consecutive negatives.

S. derby.

There were 4 infections, 3 of clinical gastro-enteritis in infants (7-15 months old) and 1 inapparent in a nurse in a maternity hospital. All occurred in October, but there was no relationship between them, unless possibly one of the babies had an intercurrent infection. He developed gastro-enteritis before the others, gave 5 negative stool examinations in the first week, and *S. derby* was not found in his faeces until a few days after the other two patients had been admitted to hospital. The infections lasted from 1 to 9 weeks, and were of moderate severity; there were no deaths. We have found no infections in animals, but Mr. Simmons has isolated *S. derby* from pigs at Toowoomba and chickens near Ipswich.

S. chester.

There were four infections with this species also. Two were examples of food-poisoning, one in a man aged 41 following a meal of mince, the other in a boy aged 3 after eating ham sandwiches (two of a family of four ate the sandwiches and became ill, the other two remained well). We did not have the opportunity to examine the food involved in either case. Both were sharp but transitory, and the boy remained a carrier for four weeks. The third human case was a mild attack of gastro-enteritis of unknown origin in a 3-months-old infant. The rat was caught in a maternity hospital kitchen. Mr. Simmons' record of this species during the same period was from pigs in a Brisbane suburb.

S. paratyphi C.

The human infection was in a healthy boy aged 3 who had had some diarrhoea six weeks previously. He was the second in a family of five children, who had contributed two proven *bovis-morbificans* infections, of whom one (a twin) died, and two cases of severe, unidentified gastro-enteritis, of whom one (the other twin) died. The discovery of a Group C infection in this boy would lead almost irresistibly to a wrong conclusion unless the organism were fully identified. The infection was transitory, and was found once in three examinations. The mother and the other two surviving children were each examined twice with negative results.

The rat was caught in a quarry adjacent to a hospital for infectious diseases. *S. paratyphi C.* has not been recorded previously in Australia, and apparently has not contributed to enteric infections in this country.

S. muenchen.

These infections illustrate how Salmonellas may enter maternity hospitals. Mrs. O. had mild diarrhoea when she entered hospital to be confined, and *S. muenchen* was found in her faeces. Four days later *S. muenchen* was found in her baby's faeces, and a week later in the faeces of another baby in the same nursery. Neither infant was sick, though their stools contained some mucus. We have seen no other infections with this species.

S. kottbus.

One inapparent infection was found in a survey of 113 mothers in a maternity hospital. Her baby showed no clinical or bacteriological evidence of infection.

S. newport.

A single infection in a three-months-old infant. At first it appeared to be mild, but after three weeks he relapsed suddenly and died. The organism was isolated from the faeces in life and the spleen after death.

S. pullorum.

This species was found only at the second of five examinations of a healthy, 15-months-old child in an institution for homeless children. It might have been acquired from egg, and apparently did little, if anything, more than simply pass through the intestine.

S. newington.

Both infections were in healthy adult carriers, one the father of a child who had been admitted to hospital with a *bovis-morbificans* infection, the other a persistent typhoid carrier, who was examined repeatedly by the hospital laboratory, and was found on one occasion to have an associated *bovis-morbificans* and on another an associated *newington*. Mr. Simmons has also found this species in chickens in southern Queensland.

S. meleagridis.

This was found once in a suburban brown rat.

S. amager.

This species, not previously reported from Australia, was isolated by the General Hospital Laboratory in November from a 12-months-old boy, who had a mild attack of gastro-enteritis, which he had evidently contracted in the temporary housing camp where he lived. Five acute *bovis-morbificans* infections and the first *newington* infection mentioned above also came from the same camp, but a survey of 33 of its inhabitants revealed nothing further.

S. sp.

Isolated from a mouse caught in a hospital ward. A known but unnamed form with the antigenic formula: XVI; k; enz_{15} (Miss Atkinson, personal communication).

DISCUSSION.

Our 10 new records, added to those previously reported, make a total of 20 species of Salmonella now known from Queensland, and indicate that the genus is well represented here. The general picture was obscured by the earlier epidemic predominance of *S. bovis-morbificans*, but the occurrence of the other species throughout the epidemic, and their continuance at about the same level since it waned, indicate that Salmonella infections contribute materially to the general incidence of infantile gastro-enteritis in the community. It is clear, too, that they cause some at least of the outbreaks of neo-natal diarrhoea in maternity hospitals. Our findings suggest also that *S. typhi-murium* will normally be found to predominate among the Salmonella infections in Brisbane, as it does in other parts of Australia.

The epidemic wave was quite unusual, not only in magnitude and severity, but in the species of Salmonella involved, and can only be regarded as an aberration from the steady endemic situation. It will be discussed in a separate paper.

Enteritic infections in the young are not all salmonelloses; some are bacillary dysentery, while many, particularly in the new-born, are still of unknown aetiology. Some of these may be due to a virus (Gorden *et al.*, 1947). It is im-

portant, epidemiologically, to distinguish between the groups, but data are few, and the proportions seem to vary more widely in different places than variations in bacteriological technique would lead one to expect.

In Uruguay, Hormaeche *et al.* (1943), for 253 children under two years old with enteritis examined in 1940–42, list 38 p.c. as bacillary dysentery, 25 p.c. as Salmonella infections, and 37 p.c. as of unknown aetiology. They show, further, that the unknown group fell with improved technique from 50 p.c. in previous years, and that it formed only 6 p.c. of the infections in children over 2 years of age. In Australia, dysentery has been well recognized since the 1914–18 war, and the methods of isolation changed not at all between the two wars; its incidence in young children has varied from 20 to 33 p.c. of cases of enteritis in different States. Recognition of Salmonella infections, on the other hand, has improved greatly, and their identification has been given precision only within the past six or seven years. Nevertheless, Harper (1932) quotes Tidswell's 356 cases in Sydney as showing 20 p.c. bacillary dysentery, 38 p.c. Salmonella infections, 42 p.c. unknown aetiology, and this remains the best comparative series to date. Draper (1945), using modern methods, records 72 infections with bacillary dysentery and 51 with Salmonella in Adelaide in 1942–44. He does not indicate the total number with enteritis examined, but makes the important point that, of 20 of the unknowns studied, 6 showed serological evidence of Salmonella infection and 6 of bacillary dysentery.

The Queensland figures for children under two years of age during the epidemic period (271 cases examined) were: bacillary dysentery 1 p.c., Salmonella 57 p.c. and unidentified 42 p.c. The "unknowns" were a heterogeneous lot, including some that were examined only once, others that were studied carefully either during life or post-mortem, some mild transitory illnesses, others so typical of salmonellosis that the clinicians had no doubts, some who were so closely associated with proven Salmonella infections in other children as to suggest the probable nature of their own illnesses, and three in whose serum Miss Atkinson found Salmonella agglutinins. We can say that there was certainly very little bacillary dysentery in Brisbane during the period, and we may feel fairly sure that we would have demonstrated more Salmonella infections if we had been able to examine more cases thoroughly; but we cannot suggest what proportion of the residue might really have been due to some other agent. Our experience might have led us to doubt the reality of the group of unknown aetiology, even in our neo-natal series, if it were not for the failure of careful bacteriological work elsewhere, like that reported by Crowley *et al.* (1941) in England and indicated by Campbell (1945) in Melbourne.

We hope to have the opportunity to examine this problem further.

So far as our proven infections were concerned, the *epidemic* was undoubtedly spread by human agency, but the sporadic distribution of the *endemic* salmonellosis was quite different and completely in accord with the generally held opinion that man is usually an accidental intruder in a normal animal-parasite association. The need for a thorough survey of Salmonella infections

in animals is obvious, and our finding that every species we isolated from rats was different is a stimulus to further work.

Finally, our experience demonstrates very clearly the importance, not only of grouping the organisms found, but of identifying them specifically. Grouping is of great provisional practical value in pointing to immediate action that may be taken during an outbreak, but we would have been led into epidemiological error on at least two occasions (by *S. paratyphi C.* and *S. muenchen*) if Miss Atkinson had not been available to give precision to our findings.

SUMMARY.

Twenty species of Salmonella are known from Queensland, of which notes are given on fourteen. One (*S. bovis-morbificans*) was associated with a grave epidemic of infantile and neo-natal gastro-enteritis in Brisbane from April to November 1947; the others, with *S. typhi-murium* predominating, were present throughout, and have contributed materially to the general level of post-epidemic intestinal infections in young children.

Acknowledgments. We are greatly indebted to Dr. J. V. Duhig and his staff, especially Mr. G. L. Facy, of the Pathology Department of the Brisbane General Hospital for a considerable number of cultures, and for permission to amalgamate their bacteriological findings with our own, to Dr. J. I. Tonge, of the Government Laboratory of Microbiology for further cultures, to Mr. G. C. Simmons for information and for agglutinating sera, and most especially to Miss Nancy Atkinson and her associates for the enormous amount of work they undertook in elucidating our problems of identification.

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THE DETECTION AND POLAROMETRIC ESTIMATION OF SULPHIDE SULPHUR IN BACTERIAL CULTURES

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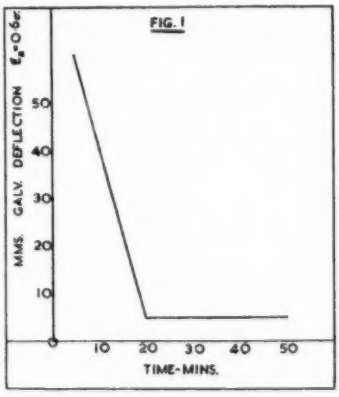
During the initial stages of our investigations on oxygen measurement with the polarograph, we employed a mercury pool as the non-polarisable anodic reference electrode (Skerman and Millis, 1948). Following the technique adopted by Baumberger (1939) we employed a washed suspension of living organisms to remove oxygen from a yeast-extract-peptone medium in order to plot the residual current curve. The method yielded consistent results and was easily applied. If, after the addition of the organisms, the oxygen concentration (in mm. galvanometer deflection) was plotted against time at a fixed applied e.m.f., a graph of the type shown in Fig. 1 was obtained. The current-time curve was a straight line. The current was rapidly reduced to a minimum value which corresponds with the residual current at this value of the applied e.m.f. From this point onwards the current remained at a constant value.



As an alternative to the use of a fresh inoculum into a fresh sample of broth we found that the use of 24-hour-old cultures of a species of *Pseudomonas* and of *Escherichia coli intermediate type I* in the same medium yielded the same results and we frequently used them for the purpose. On one occasion, however, recourse was made to the use of a similar culture of *E. coli intermediate type II* which might well have been expected to yield similar results. Instead a minimum value several millimetres larger was obtained. The cause was investigated and found to be the presence of sulphide sulphur in the latter culture. The results of subsequent investigations of the detection and determination of ionized sulphur are presented in this paper.

Examination of several organisms, including species of *Proteus* and *Salmonella,* showed that the increase in the residual current at the fixed applied e.m.f. value employed in oxygen measurement was obtained with all organisms which showed positive evidence of ionized sulphur production in yeast-extract-

peptone broth. Addition of 2–3 p.p.m. of Na_2S to the deoxygenated medium yielded similar results. Organisms which gave negative tests for ionized sulphur showed no current increase or only a slight variation.

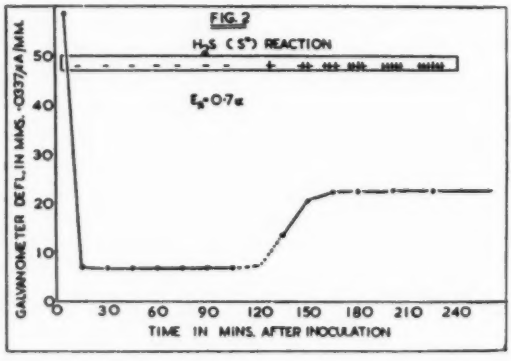
As a preliminary step in the study of the qualitative and quantitative aspects of this problem several methods were investigated for the quantitative determination of ionized sulphur. The one finally adopted, devised by Pomeroy (1936) for the determination of sulphides in sewage, is extremely sensitive and specific for sulphide ions. It depends on the interaction between dimethyl-p-phenylene-diamine hydrosulphate, ionized sulphur, ferric and chloride ions to form methylene blue. The intensity of the blue colour developed is proportional to the concentration of ionized sulphur if the other reagents are in excess, and hence can be employed in the quantitative determination of ionized sulphur. Positive results can be obtained in water for a sulphide concentration of 0·1 p.p.m. Results of the same order were obtained with the medium employed.

EXPERIMENTAL.

Correlation of Sulphide production with the change in the residual current at a fixed applied e.m.f.

If a quantity of broth is placed in the electrolysis cell using mercury pool as anode and is inoculated with a culture of *E. coli intermediate type II* to yield a final concentration of approximately 50×10^6 organisms per ml., the current-time curve plotted with an applied e.m.f. of 0·6 volt over a period of four hours appears as shown in Fig. 2.

The oxygen concentration is rapidly reduced to zero, the minimum (residual) current reading corresponding to that obtained with non-sulphide-producing organisms. The current remains constant at this value for a period and then suddenly starts to increase over a period of 10–30 minutes till it reaches a new value where it remains *relatively* constant again for a period of 24 hours.



In order to show that the current increase coincided with the production of ionized sulphur, broth was put up in a long column with a base outlet and inoculated with the suspension of *E. coli intermediate type II*. Samples were removed at 15-minute intervals to the electrolysis cell for current readings and subsequent colorimetric determinations were made for ionized sulphur on the same samples. As shown in Fig. 2 the increase in the residual current coincides with the production of ionized sulphur. As will be shown later

in this paper the current change is initiated by something less than one part per hundred million of ionized sulphur.

That the current increase was due to the production of ionized sulphur was further confirmed by the addition of known amounts of sodium sulphide, to a broth deoxygenated by an organism known to produce no sulphide. An estimation was first made of the concentration of sulphide produced by *E. coli intermediate type II* after 24 hours' growth in the medium employed. For this purpose solutions of sodium sulphide were prepared in deoxygenated water and the concentration of sulphide determined by the following method.

10 ml. aliquots of a deci-normal solution of iodine were titrated potentiometrically with deci-normal arsenious acid in the presence of excess solid sodium bicarbonate. Separate 10 ml. aliquots of iodine were acidified by the addition of 10 ml. of N/10 H_2SO_4 and 15 ml. of the sulphide solution containing approximately 0·2 gm. of the hydrated salt per 100 ml. added slowly from a pipette. The residual iodine was rendered alkaline with excess sodium bicarbonate and titrated with the arsenious acid. The sulphide concentration was determined from the difference in the amount of arsenious acid used in the two titrations.

The sulphide solution was then added to the deoxygenated broth in amounts ranging from 0–100 p.p.m. Colorimetric determinations made on these samples were compared with those made on 24-hour cultures of *E. coli intermediate type II* and *proteus*. The amount of ionized sulphur produced by these organisms was found to be approximately 5–10 p.p.m. Concentrations of this order were then used to test the effect of the sulphide on the residual current reading obtained with non-sulphide-producing organisms.

Investigation showed that the addition of sulphide to give a final concentration of 1 p.p.m. usually resulted in no current change whilst colorimetric determinations made on the same sample yielded negative results. The addition of 2 p.p.m. usually resulted in an immediate current increase to the level obtained by the presence of sulphide-producing organisms. Left for a period this current would often decrease gradually to the original zero value. It is therefore apparent that the medium is capable of "de-ionizing" the sulphide. Addition of a third p.p.m. always resulted in an immediate return of the current to the original level. Subsequent addition of up to 20 p.p.m. of sulphide resulted in only a slight change in the maximum level of rarely more than one millimetre.

This "sulphide ceiling" varied with different batches of a meat infusion broth tested, but in all instances the ceiling obtained by the addition of 3 p.p.m. of sulphide coincided with the ceilings obtained with sulphide-producing organisms.

Further evidence that it was the sulphide ion which caused the current change lies in the coincidence of the "half-wave potentials" obtained with added sulphide and sulphide-producing organisms. This is discussed in a later section of the paper.

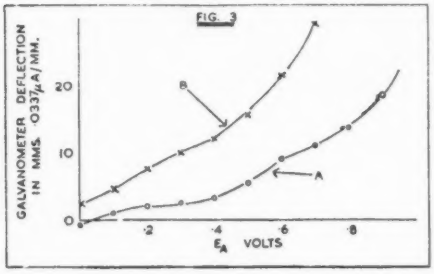
The explanation of the current increase associated with the production of sulphide lies in the use of a mercury pool as the anodic reference electrode. In a previous paper (Skerman and Millis) the relationship between the potential of the dropping electrode, on which the magnitude of the current is dependent, and the non-polarizable anode was discussed.

$$E_{cathode} = E_{anode} - E_{applied} + IR$$

In the absence of any depolarizing ions the magnitude of the current I varies only with E_c. While E_{an} remains constant E_c, and hence I, will change with any change in E_a. If, as is the case in Figs. 1 and 2, the applied e.m.f. (E_a) is held constant E_c and I will change only if E_{an} changes. Whilst the latter remains constant the current-time curve at a fixed applied e.m.f. is a straight line running parallel to the abscissa (Fig. 1 from 20–50 minutes). If anything should occur to make the anode potential more negative, E_c will also become more negative and the current I will increase. This is precisely what occurs when sulphide is produced in an oxygen-free medium in an electrolysis cell employing a mercury pool anode.

Fig. 3 shows two current-voltage curves plotted on two samples of the same meat

infusion broth, one (A) devoid of oxygen and the other (B) devoid of oxygen but containing sulphide. The potential of the mercury-pool anode in the absence of oxygen and sulphide was — 0·240 volt vs. S.C.E. In the presence of sulphide it was — 0·585 volt vs. S.C.E., that is, 0·345 volt more negative. At an applied e.m.f. of 0·6 volt, applied negatively to the dropping electrode, the potential of the dropping cathode in (A) will be —0·840 volt vs. S.C.E. whilst in (B) it will be —1·185 volt vs. S.C.E. The current I at E_a = 0·6 volt in (B) should therefore coincide with the current value obtained at E_a = 0·945 volt in (A). Examination of the curves will show that this is the case. If a current time-curve was being plotted in (A) at E_a = 0·6 v. the current would remain constant at 9 mm. Addition of sulphide would result in an immediate increase in the current to that obtained in curve B at the same applied e.m.f. (21·5 mm.).

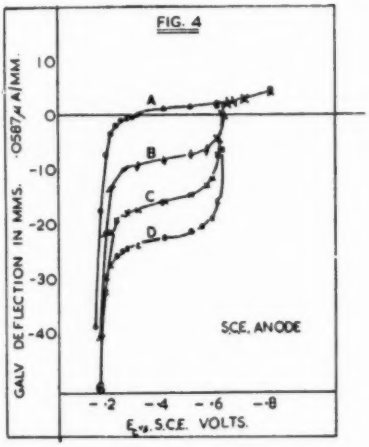


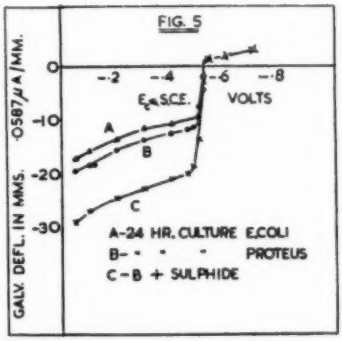
Whilst this explains the increase in current seen in Fig. 2 it does not explain why it reaches a ceiling and remains relatively constant over a long period. Quantitative determination of sulphide during the period of current increase (described in the next section) has shown that the total change in potential of the anode which occurs from the time the current begins to increase until it reaches the ceiling value is caused by a concentration of sulphide ions of the order of 0·3 p.p.m. The speed at which the potential changes is therefore dependent on the rate at which the organisms can produce this amount of sulphide. With a large population such as was employed in this experiment this time is very short. Even with a 1 p.c. inoculum of a 24-hour culture of the organism the time is somewhat less than an hour. Once the initial change in potential of the anode occurs it behaves like a sulphide electrode whose potential is dependent on the activity of the divalent sulphide ion. A ten-foid increase in the concentration of sulphide ions (from 0·3 to 3·0 p.p.m.) can be expected to change the potential only about 30 millivolts and a hundred-fold increase only 60 mvs. Thus whilst the sulphide ion concentration changes from 0·3 p.p.m. to 3·0 p.p.m. the potential of the anode will change only 0·03 volt. There will be a corresponding slight increase in the cathode potential in the negative direction. Since the residual current curve for oxygen is relatively flat such a slight change in the cathode potential will result in a very small change in the current. For this reason the current will remain relatively constant whilst the sulphide concentration changes from 0·3 p.p.m. to the 5–10 p.p.m. observed in a 24-hour culture of the sulphide-producing organism.

That the "sulphide ceiling" is reached with a concentration of sulphide of the order of 0·3 p.p.m. and remains relatively constant with changes in the sulphide concentration to 3 p.p.m. has been determined experimentally. Presentation of the result will be deferred until the polarometric determination of sulphide has been discussed.

The Polarometric Determination of Sulphide Sulphur.

If sulphide sulphur is added to oxygen-free, yeast extract-peptone broth and a *current-potential* curve plotted using a saturated calomel half-cell as anode in the electrolysis cell, curves of the type shown in Figs. 4 and 5 are obtained. It will be seen that as the cathode potential is made more positive by decreasing the applied e.m.f. from 1·0 to 0·0 volt an anodic wave is obtained starting (in Fig. 4) at $E_c = -0{\cdot}6$ v. vs. S.C.E. The wave is of a type given by a number of substances such as cyanides, iodides and sulphides, which form insoluble salts or complex ions with mercury. The theoretical aspects of these waves have been discussed by Ravenda (1934), Kolthoff and Lingane (1946) and Kolthoff and Miller (1940). The anodic wave commences when the potential has been made sufficiently positive for the concentration of the mercurous ions produced to exceed the solubility product of the insoluble salt.





With decreasing solubility the wave commences at more negative values. The actual value depends on the concentration of the anions in the solution. With increasing positive potentials beyond the potential (henceforth referred to as the "dissolution potential") at which the wave starts, the negative current increases until a limiting current is reached at which the rate of ionization of the mercury is limited by the availability of the reacting ions in the vicinity of the electrode. The depletion of the latter creates a diffusion gradient between the solution and the electrode surface so that the current becomes limited by the rate of diffusion of the anions into the depleted zone. The rate of diffusion is proportional to the concentration of the anions in the solution and hence the magnitude of the diffusion current is a measure of the anion concentration.

We have found the sulphide ion concentration in a yeast extract peptone medium at pH 6 to pH 8·0 bears a linear relationship to the increase in the negative current obtained at a cathode potential of —0·4 volt vs. S.C.E. Fig. 4 shows the current potential curves plotted after three equal additions (curves B, C and D) of a sulphide solution to broth deoxygenated with sodium sulphite (pH 7·4). Curve A is the current-potential curve in the absence of sulphide. The increments in the current with the addition of the equal amounts of sulphide do not appear proportional to the sulphide concentrations. This is due to the deionization of some of the sulphide during the period taken to plot each curve. Readings taken immediately after each addition showed current increments to be directly proportional to the sulphide concentration. Each millimetre deflection was equivalent to 0·275 p.p.m. of sulphide with the galvanometer used at 1/6 of its total sensitivity. The sharp drop at —0·2 v. is due to an anodic wave for sulphite. Addition of thioglycollate or cysteine to the medium gave waves commencing at —0·4 v. vs. S.C.E.

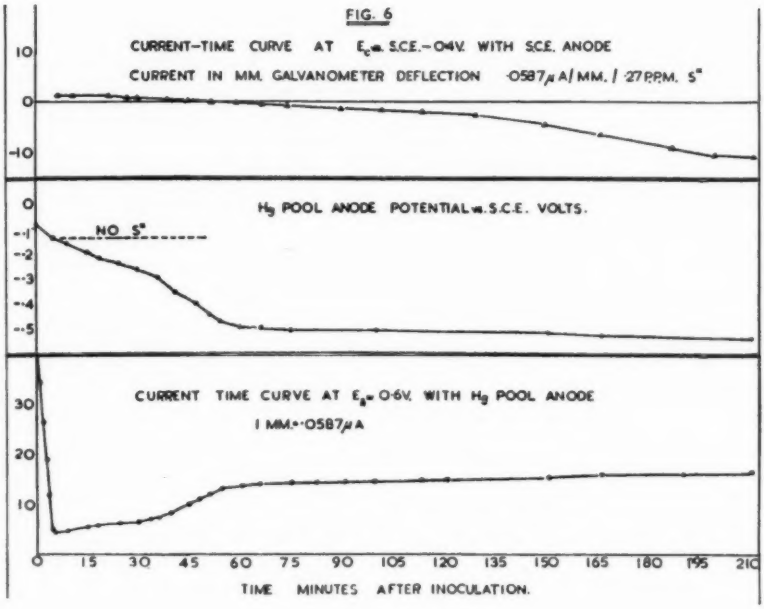
The only significant difference between the sulphide waves obtained after the addition of sulphide to the medium deoxygenated with sodium sulphite (Fig. 4) and with bacteria (Fig. 5) appears in the dissolution potential at which the wave begins. Dissolution potentials varied with different cultures examined from —·52 to —·6 v. In all instances the dissolution potential was found to coincide very closely with the potential of the mercury-pool anode in contact with the fluid. This coincidence is due to the fact that in a solution containing sulphide ions the potential that a dropping electrode, unconnected in any way with the non-polarisable anode, assumes, differs only by 3 or 4 millivolts from the potential of a quiet mercury pool in contact with the same solution. The application of a potential to the dropping electrode which is more positive than this value results in dissolution of the mercury. Variation in the value of the dissolution potential with different bacterial cultures could be due to variation in sulphide concentration or variation in the composition of the organic matter. The importance of organic matter in determining the potential of a mercury electrode has been pointed out by Skerman and Millis (1948) and has been strongly stressed by Muller (1939).

Since the dissolution potential for the sulphide wave varies from one culture to another it is necessary to check the identity of the sulphide ion by the addition of more sulphide. If the original wave is caused by sulphide the addition of more sulphide will only cause an extension of the existing wave.

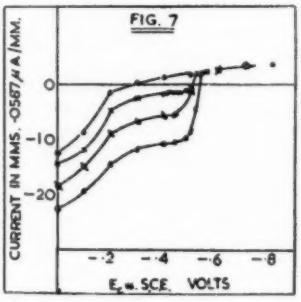
In Fig. 5, curves A and B are anodic waves obtained with 24-hour cultures of *E. coli* and *Proteus* respectively. Curve C was plotted after the addition of sodium sulphide to the *Proteus* culture after plotting curve B.

By using the polarometric method for the determination of ionized sulphur it has been possible, using the assembly described by Skerman and Millis (1948) to correlate the change in anode potential when using a mercury pool, with the current changes and with the changes in sulphide concentration during the growth of an organism.

A sample of broth was placed in the electrolysis cell and the dropping electrode and independent saturated calomel reference electrode placed in position. The applied e.m.f. (switches 4 and 8) was set at 0·6 volt. Leaving the applied e.m.f. at this value the assembly was set to measure the potential of the mercury pool anode (switches 2, 3 and 9). An inoculum of *E. coli intermediate type II* was mixed with the broth and the current and anode potentials determined simultaneously until the current reached its minimum value as in Fig. 1. A *current-potential* curve for zero oxygen concentration (the residual current curve) was then plotted using the saturated calomel half-cell as anode. Switches were set in positions 1, 3 and 9 and the cathode potential made more negative in 100 mvs. steps by increasing the applied potential. This curve was plotted in order to obtain the residual current value for sulphide change at $E_c = -0{\cdot}4$ v. vs. S.C.E. Subsequent determination of the sulphide concentration was made at this potential using the saturated calomel half-cell as anode.



Following this, determinations were made of the mercury-pool anode potential and the corresponding current at an applied e.m.f. of 0·6 v. (switches 2, 3 and 9) and of the negative current change at $E_c = -0{\cdot}4$ v. vs. S.C.E. using the saturated calomel half-cell as anode (1, 3 and 9) at intervals of time up to three and one-half hours. *Current-potential* curves using the S.C.E. anode were plotted at intervals determined by the trend of other changes. The results are shown in Figs. 6 and 7.



Examination of the Figures shows that:

(i) There is a fall in the anode potential of 54 mvs. during the removal of oxygen. The potential at 5·5 mins. (—0·140) is the potential at which the anode remains constant in the absence of sulphide.

(ii) The anode potential continues to fall at a rapid rate due to the production of sulphide, reaching a value of —0·246 v. vs. S.C.E. before there is any evidence of sulphide as determined polarometrically. (Note.—The continued fall in the anode potential almost immediately after the disappearance of oxygen is due to the use of a very heavy inoculum to reduce the over-all time of the experiment to within reasonable limits. Normally with a 5 p.c. inoculum of a 24-hour culture of the organism there is a lapse of

about one hour between the disappearance of the oxygen and the appearance of the sulphide).

(iii) The potential of the anode fell to a relatively constant value of —0·510 v. vs. S.C.E. by the time the current measured at $E_c = -0{\cdot}4$ v. vs. S.C.E. had fallen to —0·5 mm.—a difference of 1·75 mm. from the original zero of plus 1·25 mm. This is equivalent to $1{\cdot}75 \times {\cdot}275 = {\cdot}47$ p.p.m. S^{--}.

(iv) The current-time curve (lower section of Fig. 6) and the anode potential (centre section) remain relatively constant during the next 134 minutes during which time the sulphide concentration (upper section) changes from 0·47 to 3·37 p.p.m. The small change of 30 millivolts which does occur in the anode potential during the period corresponds very closely with the change one would expect from the E_h equation for a ten-fold change in concentration of the sulphide ion.

(v) Fig. 7 shows *current-potential* curves plotted at intervals of 10, 105, 157 and 202 minutes respectively after the addition of the inoculum. The dissolution potential for the successive waves becomes more negative with the increase in sulphide concentration. In each case the dissolution potential coincides with the mercury pool anode potential at the same time interval.

DISCUSSION.

The evidence presented in this paper shows that the concentration of sulphide ions and their rate of production by bacteria can be determined quite simply by a polarometric technique, using an electrolytic cell with a saturated calomel half-cell as anode. By incorporating a mercury pool and platinum contact in the cell one can obtain a very sensitive indicator of the instance at which free ions appear in the medium and this serves as a guide for polarometric measurement of the subsequent rate of production. It is apparent from these and other unpublished experiments that sulphide ions are not produced while oxygen is present in the medium. This is what one would expect from our present conceptions of oxybiontic and anoxybiontic metabolism. Whether sulphide measurements made in this manner can be taken as an accurate measurement of the total sulphide sulphur produced is questionable since it has been shown that addition of 1 p.p.m. of sodium sulphide to the yeast extract medium employed in these studies resulted in immediate de-ionization. But there is a difference between the condition under which this observation was made and the condition under which the polarometric measurement of sulphide production by the growing organism was performed. In the former the sulphide was added to medium immediately after the removal of oxygen by a non-sulphide-producing organism when the medium would still be in a relatively high state of oxidation. In the latter it is probable that the production of sulphide does not occur until this "relatively high state" of oxidation has been considerably reduced. That is, the sub-

stances with which the added sulphide reacted may not be present in the medium when the sulphide is produced by the growing organism. In this case the measurement of sulphide concentration may be an accurate measurement of all sulphide sulphur produced.

It is also interesting to note that the curve showing the sulphide concentration in Fig. 6 is beginning to flatten out at the 195-minute interval. The actual time taken for the curve to flatten out completely has not been determined but it appears certain that the reducible sulphur compounds in the medium are attacked and completely reduced by sulphide-producing organisms soon after the removal of oxygen. This sulphide contributes markedly to the fall in potential of the culture as measured with a platinum electrode.

SUMMARY.

The polarometric measurement of ionized sulphur in a bacterial culture has been discussed.

An apparatus was devised which permits the study of oxygen consumption and sulphide production rates in growing cultures of bacteria.

The change in potential of a mercury pool electrode in contact with the solution acts as a very sensitive indicator of sulphide ion production.

The significance of the polarometric measurements has been discussed.

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THE APPLICATION OF THE POLAROGRAPH TO THE DETERMINATION OF OXYGEN CONCENTRATION AND OXYGEN CONSUMPTION RATES IN BACTERIAL CULTURE MEDIA

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The polarograph is becoming an increasingly important tool in biological research. One of its many applications is in the determination of oxygen in fluids. Baumberger (1939) employed it in a study of respiration in yeasts; Peterings and Daniels (1938) in studying respiration in algae and Ingols (1942) in studying the rise and fall of oxygen concentrations in sewage. We are making use of the instrument to study the relationship between oxygen concentration and denitrification by certain denitrifying organisms.

It would be impracticable here to outline all the pitfalls one may encounter in the initial use of a polarograph. It must be emphasized, however, that a thorough knowledge of the theory of polarographic measurements is essential for the successful use of the instrument. For those unfamiliar with the instrument a brief outline on polarographic analysis by Kolthoff and Laitinen (1941) and a series of articles by Muller (1941) are recommended for preliminary reading. A list of the most important points for general consideration is given by Kolthoff (1942).

In this paper we propose to discuss the selection of electrolysis cells suitable for oxygen determination in respect to the type of anodic reference electrode employed; the "one-potential" and "two-potential" methods used for the determination of oxygen concentrations and a modified technique for the determination of oxygen in organic fluids suitable for use in calibrating the polarograph for oxygen determinations in solutions containing organic matter.

The Choice of Anodes in Preparing Electrolysis Cells for the Polarographic Determination of Oxygen Concentrations.

Two different types of anodic reference electrodes have been employed in cells used for oxygen determinations. One, recommended by Lingane and Laitinen (1939), is a saturated calomel half-cell separated from the main electrolysis cell by an agar bridge saturated with potassium chloride. Its potential remains constant at the constant temperature of the thermostat and is independent of the nature of the solution being electrolysed. This type has been employed by most workers. The other, commonly employed in inorganic analysis

and used by Peterings and Daniels (1938) in studying the respiration of algae, is a mercury pool present in the electrolysis cell in contact with the solution being electrolysed. The potential of the pool is dependent on the composition of the solution. Organic materials exert a predominating effect in determining the potential of the pool if they are present.

The normal procedure in polarographic analysis is to plot the current against the applied e.m.f. to obtain a current-voltage curve. It is extremely important that the potential of the anode reference electrode should remain constant with changing values of the applied e.m.f. if this procedure is followed.

Selection of Apparatus.

Descriptions of manually operated instruments which are ideally suited to the study of oxygen consumption are obtainable from practically every paper dealing with the subject. A diagram of the set-up employed in this laboratory is shown in Fig. 1. It was designed to study the use of the different anodic reference electrodes and to enable studies to be made on growing cultures.

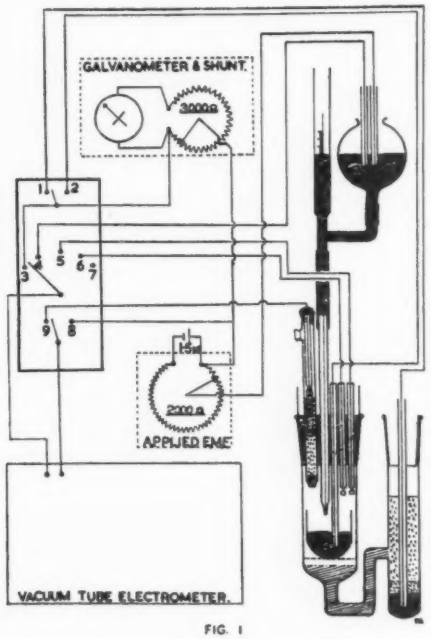


FIG. 1

The source of applied e.m.f. is a 1·5 v. dry cell connected in parallel with a 2,000 ohm variable resistance which acts as a potential divider. The positive terminal is connected to a universal shunt with a total resistance of 3,000 ohms which is connected in parallel with the current recording galvanometer whose internal resistance is 351 ohms. The negative terminal is connected to the dropping electrode.

The cell is a type recommended by Lingane and Laitinen (1939) modified to suit materials available. The calomel electrode tube (A) was made from a B19 Quickfit socket and the platinum contact was sealed into a glass tube mounted in a rubber bung inserted into a B19 Quickfit cone. The electrolysis cell consists of a B29 Quickfit socket sealed on to the reduced stem of a sintered glass mercury filter. The tapered stem was bent round and sealed into the side of the calomel electrode tube. This connecting tube was filled with 2 p.c. agar saturated with potassium chloride by applying a partial vacuum to the electrolysis cell and pouring the molten agar into the calomel electrode tube. The latter was subsequently washed and the electrode prepared.

The dropping mercury electrode capillaries have been prepared from thermometer tubing and connected to the mercury reservoir by Nylex plastic tubing. This has been employed in place of the more common rubber tubing and has given excellent results. It is important to select a tubing with a slightly smaller bore than the outer diameter of the capillary. Softened

in hot water it can be easily slipped on and will cool firmly in position. The capillary is maintained in a vertical position by passing it through a closely fitting hole bored in a polystyrene plug inserted inside a B29 Quickfit. Constant height is maintained by means of a metal clip (not illustrated) fixed to the upper part of the capillary stem, and resting on the upper edge of the glass cone. The capillary can be fixed firmly in position by moistening the inner surface of the hole with carbon tetrachloride before inserting the capillary.

To prevent mercury drops covering the sintered glass disc a small glass cup is inserted in the cell. A second reference electrode is made available if a platinum contact (connected to switch 2 in Fig. 1) is placed in a mercury pool in this glass cup. This arrangement made possible a direct comparison of the mercury pool anode with the saturated calomel anode using the same sample of solution in the cell.

The platinum electrodes connected to switches 5 and 6 are intended for redox potential measurements. They do not form part of the polarographic circuit.

The fifth electrode, connected to switch 9, is an accurately calibrated saturated calomel half-cell (Eh = ·246 v. at 25° C.) of the type supplied with the Jones Model B pH electrometer for titration purposes. Contact with the solution is by means of a sintered glass disc and a column of saturated KCl in place of the usual agar bridge.

Measurement of Potentials.

Since the external e.m.f. is applied through an uncalibrated potential divider it is necessary to measure the applied e.m.f. with a suitable measuring instrument. The vacuum tube potentiometer in the Jones pH electrometer was employed for this purpose. It has a range of 0 to 1·3 v. and has been found satisfactory for oxygen measurements. By means of the simple switch-board it has been possible to determine the applied e.m.f. and the potentials of the different electrodes with reference to the saturated calomel electrode in the switch positions indicated: applied e.m.f. (4 & 8); potential of the dropping electrode (4 & 9); redox potential (5 & 9, 6 & 9) saturated calomel reference electrode (1, 3 & 9) and the mercury pool reference electrode (2, 3 & 9).

The use of the vacuum tube potentiometer permits the measurement of the potential of the dropping mercury electrode (d.m.e.) and the anodes whilst an e.m.f. is applied across the cell, without affecting the current reading on the galvanometer.

The two independent anodes were incorporated in the electrolysis cell illustrated in Fig. 1 with the object of determining their suitability in oxygen measurement in solutions containing organic matter. It is possible with the apparatus to plot the current against the applied voltage and also against the potential of the dropping electrode for a single sample using the two anodes in turn.

A sample of broth containing 0·5 p.c. Difco yeast extract; 1 p.c. Difco peptone and 0·5 p.c. NaCl in distilled water was placed in the cell and inoculated with a suspension of *E. coli*. After the removal of oxygen the current was plotted against the applied e.m.f. (E_a) and the potential of the d.m.e. (E_c) using first the S.C.E. anode and later the mercury pool anode. The results are shown in Table 1.

TABLE 1.

	Anode					
	Saturated calomel half-cell			Mercury pool		
E_a	E_c	E_{an}	C	E_c	E_{an}	C
0	0	0	—10	—149	—142	—1·75
100	—96	0	—6·5	—242	—142	0·5
200	—195	0	0·0	—345	—142	1·5
300	—296	0	1·25	—446	—142	2·0
400	—395	0	1·75	—546	—142	2·75
500	—496	0	2·5	—646	—142	3·75
600	—595	0	3·5	—746	—142	5·5
700	—696	0	4·5	—846	—142	7·25
800	—797	0	6·5	—948	—142	9·75
900	—896	0	9·5	—1,042	—142	14·0
1,000	—994	0	11·75	—1,138	—138	18·75

E_a = applied e.m.f.; E_c = cathode potential (of d.m.e.); E_{an} = anode potential; C = current in millimetres galvanometer deflection (1 mm. = ·0337 u A).

All measurements in millivolts. Potentials referred to the saturated calomel electrode at 25° C.

The magnitude of the current measured with the galvanometer is dependent on the potential of the d.m.e. If the latter is polarized cathodically the relationship between its potential, the potential of the non-polarizable anode, the applied e.m.f. and the current is given by the equation:

$$E_{cathode} = -E_{applied} + E_{anode} + IR \qquad (1)$$

where I is the current and R the resistance.

Thus if the applied e.m.f. is 0·6 v. and the anode potential is — ·142 v. vs. S.C.E. the potential of the cathode will be equal to (— 0·742 v. + IR) volts vs. S.C.E.

The potential of the d.m.e. is therefore *initially* determined by the potential of the anode *in the absence of any applied e.m.f.* and since the current is dependent on the potential of the d.m.e. different current values will be obtained for the same solution in the absence of any applied e.m.f. if two different anodes are employed. This is illustrated in Table 1.

With an increase in the applied e.m.f. the potential of the dropping electrode becomes more negative whilst that of the anode remains constant. At the same time the current becomes more positive. This is also illustrated in Table 1.

The equation also indicates that if the same solution is electrolysed in two cells having different anode potentials but employing the same or similar dropping electrodes the same current value will be obtained for the same potential of the d.m.e. in each case provided the circuit resistance R is the same in both cases. In order to obtain the same cathode potential in each case *different values* of the applied e.m.f. must be used. This is again well illustrated in Table 1.

It is customary in polarographic analysis to plot the current against the applied e.m.f., the resulting curves, shown in Fig. 2, being termed by Muller "current-voltage" curves. Such curves are quite suitable for quantitative analysis since in the latter the wave height is the important factor and it is independent of the half-wave potential. They are not suitable for exact qualitative analysis since the identification of an ionic species is dependent on its half-wave potential. Since the latter is the potential of the dropping electrode at which the activities of the oxidized and reduced ions at the electrode surface are equal it is necessary for accurate qualitative analysis to plot the current against the potential of the dropping electrode instead of against the applied e.m.f. Such curves are termed by

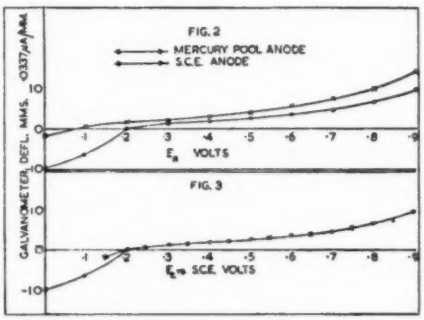
Muller "current-potential" curves. They are equally useful for quantitative analysis. In practice these current-potential curves may be obtained by two methods. One, employed by Kolthoff (1939) and used in our studies, is to determine the potential of the d.m.e. against an independent standard reference electrode such as the saturated calomel electrode, and to plot the current against the known cathode potentials. The second, discussed by Muller (1941) is to plot a "current-voltage" curve; determine the resistance of the circuit (R); from the values of the current (I) obtained for each value of the applied e.m.f. (E_a) calculate the values of IR for each value of E_a and finally plot $E_a - IR$ against I for each value of E_a.

Referring to equation (1)

$$E_c = E_{an} - (E_a - IR)$$

since E_{an} is a constant, plotting of the values of $E_a - IR$ against I (Muller) will yield a curve identical with that obtained by plotting E_c against I (Kolthoff), that is, they are both "current-potential" curves within the meaning of the term as defined by Muller. But the half-wave potentials obtained from the two curves are not directly comparable since the potential of the dropping electrode as determined by Kolthoff may have been referred to an electrode or half-cell with a different potential from that of the anode employed in the cell —the reference electrode employed by Muller.

If instead of plotting the values of $E_a - IR$ against I the whole value $(E_{an} - [E_a - IR])$ is plotted against I for each value of E_a the half-wave potentials would be directly comparable with those obtained by plotting E_c against I provided E_c and E_{an} are both determined with reference to the same electrode. This would entail, in the method described by Muller, the additional measurement of the anode potential (E_{an}).



In addition, if the electrolyses are carried out in a number of cells with different anode potentials the use of Muller's method would entail the determination of the anode potential and R of each cell if half-wave potentials are to be directly compared. By plotting current potential curves by Kolthoff's method all current values will be the same over identical ranges of cathode potentials irrespective of the potentials of the anodes. This is illustrated in Figs. 2 and 3. In Fig. 2 current-voltage curves for the deoxygenated yeast extract peptone medium are shown using two different reference anodes —a mercury pool with a potential of —·142 v. vs. S.C.E. and a saturated calomel half-cell. In Fig. 3 current-potential curves with both anodes are found to coincide.

In normal practice the values of I and R are very small so that for small currents of 3 or 4 microamperes the product IR may be negligible. In such cases the values of $E_a - IR$ approach E_a and the current-voltage and current potential curves may be almost identical.

The general advantage of plotting the cathode potential as determined by Kolthoff against I disappears if, as anode, a saturated calomel reference electrode is employed as recommended by Lingane and Laitinen. In this case the equation

$$E_c \text{ vs S.C.E.} = E_{an} \text{ vs S.C.E.} - (E_a - IR)$$

becomes

$$E_c \text{ vs S.C.E.} = 0 - (E_a - IR).$$

If IR is negligible the current-voltage curve obtained by plotting E_a against I will be equal to and directly comparable with that obtained by plotting E_c vs. S.C.E. against I (cf. the current-voltage and current-potential curves obtained with the S.C.E. anode in Figs. 2 and 3).

It would appear from Table 1 that the mercury pool anode employed by Peterings and Daniels satisfies the requirements in polarographic analysis, its potential remaining constant for all values of the applied e.m.f. This has been found to be so only in the absence of oxygen at definite pH values. In the presence of oxygen its potential becomes more positive with increasing applied e.m.f.

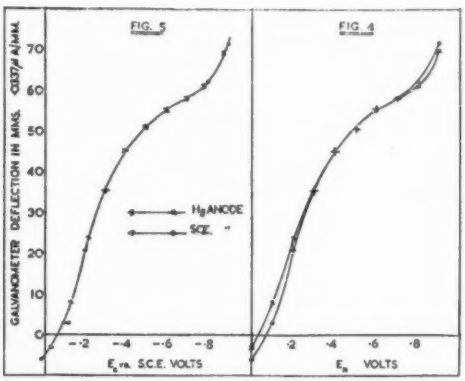
Table 2 shows the results of an experiment in which the applied e.m.f. (Fig. 4) and cathode potentials (Fig. 5) were plotted against current using the yeast extract peptone medium containing approximately 7 p.p.m. oxygen.

TABLE 2.

	Anode					
	Saturated calomel half-cell			Mercury pool		
E_a	E_c	E_{an}	C	E_c	E_{an}	C
0	10	10	—6·6	—30	—28	—3·0
100	—90	10	3·0	—122	—20	8·0
200	—192	10	20·75	—208	—8	23·75
300	—288	10	35·5	—290	8	35·5
400	—386	10	45·25	—383	12	45·0
500	—486	10	50·75	—480	18	50·5
600	—586	10	55·25	—580	20	55·5
700	—686	10	58·25	—680	15	58·0
800	—786	10	62·25	—766	28	61·25
900	—886	10	71·5	—860	37	69·5

For Legend see Table 1.

It will be seen that the mercury pool anode potential changes a total of 65 mvs. between applied e.m.f's of 0 to 0·9 v. Comparison of these potentials with that of the anode in the absence of oxygen shows that there is a fall of over 100 mvs. in the anode potential between oxygen saturation at 25° C. and zero oxygen concentration at the same temperature. The potential of the saturated calomel anode, although differing a little from the standard electrode owing to impurities in the cell, remains constant over all ranges of applied e.m.f's. It is obvious that the mercury pool anode potential varies both with the applied e.m.f. and the oxygen concentration. It is, therefore, exceedingly unreliable if the current is plotted against the applied e.m.f. It yields, however, results identical with those obtained with the saturated calomel anode if current is plotted against the cathode potentials (see Figs. 4 and 5). This is easy to understand when one considers that any change in the potential of the anode will be immediately reflected in the potential of the cathode.



Effect of pH on the Potential of the Mercury Pool Anode and the Current in a Yeast Extract Peptone Medium Devoid of Oxygen.

The value of the residual current at a certain value of the applied e.m.f. is important in the quantitative determination of oxygen. The effect of pH on the residual current was therefore determined, using both anodes, for a single value of applied e.m.f.—that employed in oxygen measurements with our apparatus.

Samples of the medium were placed in the cell and deoxygenated with a living suspension of *E.coli*. The pH was adjusted by addition of small amounts of N/1 NaOH and N/1 HCl. Current measurements were made at an applied e.m.f. of 610 mvs. The results are shown in Table 3.

TABLE 3.

	Anode						
	Saturated calomel half-cell				Mercury pool		
pH	E_a	E_c	E_{an}	C	E_c	E_{an}	C
8·39	610	—600	10	3·0	—775	—170	5·0
6·23	610	—600	10	2·5	—744	—142	4·5
5·98	608	—600	10	2·5	—752	—142	5·25
5·54	608	—600	10	3·0	—751	—142	5·75
4·65	610	—600	10	3·75	—748	—135	7·5
4·55	610	—600	10	4·0	—730	—115	7·75
4·08	610	—600	10	6·75	—712	—100	9·5
3·70	608	—600	10	11·5	—665	—170	13·5

For Legend see Table 1.

It will be seen that the residual current remains relatively constant between pH 5·5 and 8·4—the principal biological pH range. At pH values below 5 the residual current increases sharply. This is the case with both anodes but is modified with the mercury pool anode due to a change in the potential of the anode itself with changes in pH values.

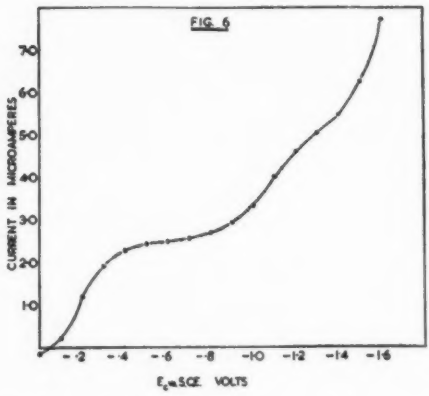
The Measurement of Oxygen.

A Comparison of the One-potential and the Two-potential Methods Employed in Oxygen Determinations.

Fig. 6 shows the *current-potential* curve obtained for oxygen at pH 6·6 in the yeast extract peptone medium. The waves are very similar to those illustrated by Baumberger (1939) and Hartmann and Garrett (1942), but differ markedly from those published by Peterings and Daniels (1938). The first wave, ceasing at about $E_c = -\cdot 7$ volt vs. S.C.E. is the one normally employed in oxygen measurement. The curve is in no place entirely flat, the first wave merging almost immediately into the second. The point of inflection at $E_c = -0\cdot 6$ volts vs. S.C.E. was selected as wave height. The wave was plotted with the medium at 25° C. and a drop rate of 2·7 secs.

The concentration of oxygen should be directly related to the difference (i_d) in current between the current (i) obtained at a potential of 0·6 volt in the medium containing oxygen and the residual current (i_r) obtained at the same potential in the absence of oxygen.

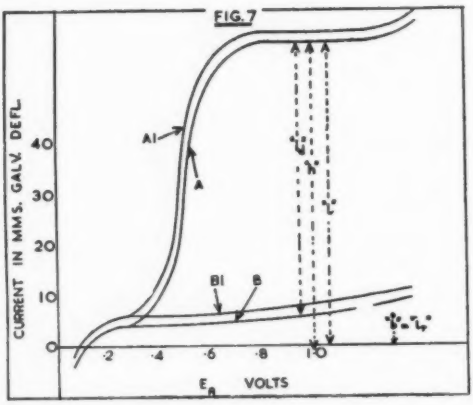
$$i_d = i - i_r$$

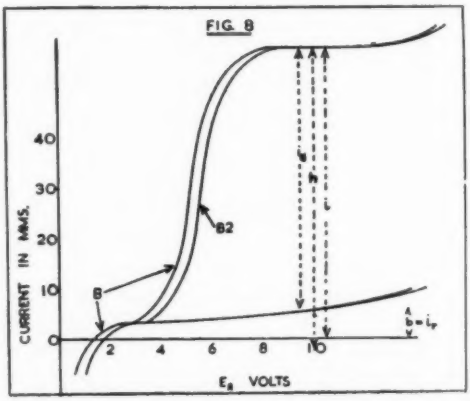


The removal of oxygen for the determination of (i_r) can be achieved by a number of methods. The principal ones involve removal by means of a stream of oxygen free hydrogen or nitrogen; addition of living cultures of yeasts or bacteria or the addition of 1·0 p.c. of sodium sulphite. In our experience they all yield the same result at the same pH with the same dropping electrode coupled with either the saturated calomel electrode or mercury pool anode at $E_c = -0{\cdot}6$ v. The values differ however, if they are determined at a fixed value of the applied e.m.f. due again to difference in the anode potentials.

The One-potential Method.

Let us suppose that curve A in Fig. 7 represents the current voltage curve obtained for oxygen in an unspecified medium and that curve B (Fig. 7) represents the residual current curve plotted with the same medium devoid of oxygen. The limiting current (i) is reached at 0·8 v. and remains constant over the range 0·8 to 1·2 v. The residual current at 1·0 v. is "i_r". The oxygen concentration in this solution is directly proportional to the difference





"i_d" between "i" and "i_r". If different oxygen concentrations are plotted against their corresponding values of "i_d" the resulting curve will be a straight line with the equation

$$y = x\ i_d$$

where y is the oxygen concentration; x is the oxygen coefficient in parts per million (or other unit) per microampere (or millimeters galvanometer deflection) depending on whether i_d is expressed in microamperes or millimeters galvanometer deflection.

For most practical purposes, therefore, it is only necessary to determine the oxygen concentration for one value of i_d in order to derive the coefficient, although it is advisable when working with a solution for the first time to make several determinations. When determining the oxygen concentration in an unknown solution "i" and "i_r" are determined at the *one potential*, the difference between the two yielding "i_d" which is simply multiplied by the oxygen coefficient.

The Two-Potential Method.

This method was employed by Peterings and Daniels (1938). In this method two readings are made *on the solution containing oxygen*, one in the region of the limiting current potentials (0·8 to 1·2 v. in Fig. 7) and another at some potential preceding the decomposition potential of oxygen. For the purpose of illustration these two potentials are taken on curve A (Fig. 7) at 1·0 and 0·1 v., the current difference being represented by "h". If oxygen concentrations are plotted against the corresponding values of "h" the resulting curve will again be a straight line but with the equation

$$y = x\ (h - n)$$

where "n" is the difference between "h" and "i_d".

To derive the value of "x" the value "i_r" at the higher potential must therefore be known in order to obtain "i_d". An oxygen coefficient is not normally derived in the two potential method, the oxygen concentration being obtained by direct reference to a graph.

From the point of view of simplicity the two-potential method is undoubtedly the easier method to employ but the validity of the method is obviously dependent on perfect reproduction of the residual current curve for all samples of the solution under all conditions. This obviously cannot be the case where oxygen measurements are being made with a mercury pool anode which has been shown to change its potential with changes in oxygen concentration, applied e.m.f. and pH.

In supporting the use of the two-potential method Peterings and Daniels stated that "it might be argued that with this calibration method a single voltage rather than two voltages would be sufficient, but the zero point of the galvanometer fluctuates with temperature and accidental variations in the amount of iron and other easily reducible substances give large fluctuations even at 0·1 v. These fluctuations are cancelled out by taking the difference between two voltages".

It can be seen by reference to Fig. 7 that the final statement does apply to a change in the galvanometer zero brought about by a mechanical disturbance or change in temperature. The significance of curves A and B have already been discussed. If the galvanometer zero changes by 2 mm. after these curves have been plotted and the curves are replotted, they assume the positions A1 and B1 respectively. The value of "h" does not alter although "i" alters by 2 mm. Whilst the statement is therefore correct it does not mean that the use of the one-potential method would yield an erroneous result. Although "i" alters by 2 mm. "i_r" alters by the same amount so that "i_d" remains constant.

The statement does not apply however when changes in the residual current are brought about when "iron and other easily reducible substances" are present in the medium. This

is particularly the case where a mercury pool anode is employed but applies also to the use of the saturated calomel half-cell as anode.

A small trace of sulphide may alter the potential of a mercury pool quite considerably. If the same pool is used during the electrolysis of several solutions containing oxygen, one of which contains a small trace of sulphide, the potentials of the pool before and after contact with the sulphide may differ by 100 mvs. due to a residual effect of the sulphide. In Fig. 8 current voltage curves for oxygen B and B2 are shown plotted before and after contact with sulphide. The residual current curves are also illustrated. It can be seen that the change of 100 mvs. in the anode potential results in a change of 3 mm. in the reading taken at 0·1 v. without any alteration in the reading taken at 1·0 v. By using the two-potential method an incorrect value would be obtained for "h" whilst application of the one-potential method would yield the correct value for "i_d". It will be seen from a subsequent paper that the objection to the use of the two-potential method applies equally as well when a saturated calomel half-cell is employed as the anode.

The two-potential method cannot therefore be considered generally valid. Its use is extremely inadvisable when studying processes in which living organisms are present in fluids containing organic matter since changes in the composition of the organic matter are inevitable.

The Chemical determination of Oxygen for the Calibration of the Polarograph.

It is necessary to calibrate the polarograph for the quantitative determination of any reducible or oxidisable material. This is comparatively simple with metals and other substances, solutions of which can be prepared with a high degree of accuracy. The calibration for oxygen is not so simple since the initial chemical estimation of dissolved oxygen is itself not easy. Two direct methods can be adopted, their suitability being determined by the substrate in which the oxygen is dissolved. The Winkler method for dissolved oxygen in water is suitable for synthetic media devoid of organic matter or only containing a small quantity. It is not suitable for the determination of oxygen in solutions containing a high concentration of organic matter. For the latter, the method devised by Sharp, Hand and Guthrie (1941) and employed by Hartman and Garrett (1942) has proved satisfactory. We have modified this method in a manner which makes it more suitable for the calibration of the polarograph and eliminates some of the manipulations in the Sharp method which are liable to lead to error. The special tubes employed by Sharp *et al.* were discarded and all manipulations carried out in the polarographic cell. The only special requirement for the modified method is that the cell should be large enough to hold about 30–40 ml. of the medium and that the capillary tip should reach at least to the centre of the cell. These requirements were satisfied by the cell we have employed in our studies.

If a small cell is employed in normal routine estimations and a larger cell is temporarily used in order to apply this method, care should be exercised that all normal standards, particularly in regard to the mercury column height are observed.

The dropping electrode is removed from the cell and 30 ml. of the medium introduced. There is no necessity to take any of the special precautions in introducing the material that are required in the filling of the Sharp tube. The broth is covered with a layer of freshly boiled and cooled paraffin oil. The dropping electrode is then introduced through the oil and set in position. Provided this is done quickly there is no interference with the behaviour of the capillary. The fluid is left untouched for 10 minutes and then the current reading (i) is taken at the selected cathode or applied potential. The electrode is then removed and a glass stirring rod introduced through the oil. The required quantity of concentrated ascorbic acid solution is then pipetted into the broth and the mixture immediately stirred. One or two 5 ml. aliquots are then removed with a minimum of delay and transferred to the 15 ml. quantities of N/10 sulphuric acid.

One half millilitre of *freshly expressed* cucumber juice is then mixed with the broth and the dropping electrode replaced. The two 5 ml. samples can then be titrated with the standardized dye (2–6 dichlor-phenol indophenol) while the enzymic reaction is taking place in the cell. The rate of the current fall at the selected cathode or applied potential can be followed on the galvanometer till the residual current value is reached. This value should be determined by a preliminary titration. A period of two minutes can then be allowed to ensure that the current has reached a constant minimum. A number of 5 ml. samples are then removed and transferred to 15 ml. amounts of N/10 sulphuric acid and titrated against the standardized dye. The subsequent estimation of the oxygen concentration is the same as that employed by Sharp *et al.* with the exception that the ·45 mg. litre correction is not applied since the reasons given for its use are not considered to apply in this method. The "oxygen coefficient" can then be obtained by subtracting the residual current reading from the initial oxygen reading and dividing this value into the oxygen concentration to obtain the oxygen coefficient in p.p.m./mm./galvanometer deflection.

There are two principal sources of error in the method as described. The first is in the choice of the residual current value for subtraction from "i" to obtain "i_d". One would feel tempted to use the zero value for oxygen obtained after all the oxygen has been exhausted in the oxidation of the ascorbic acid. This is not justifiable unless a correction is made for the change in the residual current which accompanies any change which may occur in pH after the addition of the ascorbic acid since the values for "i" and "i_r" may have been obtained at two different pH values. If the medium is well buffered or the current change with pH is negligible this correction can be ignored.

The second source of error arises when the determination of the initial concentration of ascorbic acid is made. The oxygen concentration is estimated from the difference between this value and the value obtained after the oxygen has been exhausted. This oxygen value does not necessarily correspond with the value for which "i_d" was originally determined, since a small amount of the ascorbic acid will be oxidized in the interval of time between the addition of the ascorbic acid and the taking of the initial samples (i.e. during the period of mixing). Sharp *et al.* made no attempt to apply a *specific* correction for this but used an over-all correction for errors during manipulation which appears to have no concrete basis. By the modification described in this paper this error can be simply estimated and, if necessary, eliminated. This can be done by taking note of the time interval between the addition of the ascorbic acid and the initial sampling and making a separate determination of the rate of oxygen consumption (in mm. per minute) in the presence of the same concentration of ascorbic acid as that employed in the titration. From these values one can calculate the change in ("i") which would have occurred during the time interval. This subtracted from the original value of "i" will give the true value of "i" at the time of sampling. Subtraction of "i_r" will then yield the true value of "i_d" for the concentration of oxygen determined by the ascorbic acid titration.

The method offers an obvious advantage over the determination of oxygen in the Sharp tube and the separate determination of "i_d" in the polarographic cell—the method employed by Hartman and Garrett. The necessity to saturate the sample with air or to handle the samples carefully when not saturated to avoid agitation are eliminated. Direct observation of the current fall eliminates the necessity to employ a concentrated enzyme preparation and ensures that the residual ascorbic acid is estimated immediately after the oxygen has been exhausted.

It is necessary, of course, in using the instrument for a subsequent oxygen determination in the same medium, to introduce the fluid into the cell with the maximum of care if the actual value is required.

Application of the method has shown that the relationship between the oxygen concentration and current "i_d" is linear. This is in full agreement with the findings of Hartman and Garrett with milk.

DISCUSSION.

The polarograph can be a useful tool for studying oxygen consumption by micro-organisms and other agents provided due attention is paid to the limitations of the method. The most useful type of cell is one which employs the saturated calomel half-cell as anode, as recommended by Lingane and Laitinen. If this type is used practically nothing can be gained by plotting current-potential curves in place of the more usual current-voltage curves.

Whilst it has been demonstrated that plotting of current-potential curves eliminates the more undesirable traits of the mercury pool electrode and makes possible the qualitative and quantitative determination of oxygen with the same degree of accuracy as that obtained with the saturated calomel anode, the use of the mercury pool anode is not to be recommended for studying oxygen consumption by bacteria in organic fluids since marked changes in the composition of the medium due to bacterial action may bring about sharp and large changes in the potential of the mercury pool anode and make subsequent measurements of oxygen impracticable. One instance of this will be discussed in a subsequent paper. In this connection it is important to draw attention to the fact that the potential of the mercury pool anode in the absence of oxygen shown in Table 1 (—0·142 v. vs. S.C.E.) is approximately 250 millivolts more negative than a calomel half-cell with the same chloride ion activity as that present in the medium. It is obvious that the potential is determined principally by the organic constituents of the medium.

SUMMARY.

The use of a saturated calomel half-cell and a mercury pool as anodes in a polarographic cell for the measurement of oxygen in solutions containing organic matter has been investigated.

The potential of the mercury pool anode was found to vary with the oxygen concentration and the applied e.m.f. and also with the pH of the medium.

Nevertheless, the variations can be compensated for by plotting the current against the potential of the dropping electrode instead of the applied e.m.f.

The validity of the two-potential method for determining the true diffusion current has been discussed.

A modification of the Sharp ascorbic acid method for determining oxygen in solutions containing organic matter is described.

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THE SIGNIFICANCE OF PROTHROMBIN ACCELERATOR IN THE THROMBIN FORMATION BY RUSSELL VIPER VENOM

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Since Martin in 1893 recognized that certain snake venoms have the property of promoting blood coagulation, this phenomenon has been studied by various workers. Eagle (1937) considers that the activating mechanisms fall into two groups. In one it appears that fibrinogen is acted upon in a manner similar to that observed with certain proteolytic enzymes whilst the other is comprised of venoms capable of converting prothrombin to thrombin in the same way as that found with thromboplastic substances isolated from mammalian sources. It is generally agreed that Russell Viper venom falls into this category and its use has been suggested by Fullerton (1940) and Page and Russell (1941) for the assay of prothrombin. In addition, Trevan and Macfarlane (1936), Leathes and Mellanby (1939) and Edsall (1941) have shown that the action of viper venom can be enhanced by the addition of lipids, and a procedure using egg lecithin in addition to viper venom has been used by Witts and Hobson (1940) for the estimation of prothrombin.

A comparison of the results obtained by the use of the various types of thromboplastin reveals certain differences. The combination of Russell Viper venom and lipid gives very similar clotting times with a variety of mammalian plasmas, suggesting similar prothrombin concentration, Quick (1945), and Fantl and Nance (1948). On the other hand, by the use of homologous brain extract Quick (1941) found markedly different prothrombin times. Finally, it is a common observation that in cases of apparent low prothrombin activity, as indicated by the use of organ extracts, the Russell Viper venom + lipid combination very often shows higher prothrombin levels, De Beer (1947); Fantl and Nance (1947a). With the recent demonstration that the rate of thrombin formation depends, in addition to prothrombin concentration, on the presence of prothrombin accelerator (Fantl and Nance, 1946), which is probably identical with Owren's factor V (1947) and Honorato and Quick's labile factor (1947), an investigation of the action of Russell Viper venom on thrombin formation on the isolated components of the clotting system has been carried out.

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EXPERIMENTAL.

Methods for the Preparation of Substances.

All prothrombin preparations were made from cell-free oxalated human plasma by adsorption on barium carbonate or barium sulphate, the details for which have been given previously (Fantl and Nance, 1948). When barium sulphate was used, elution was carried out with 0·066 M Sörenson phosphate buffer, pH 7·8.

Although prothrombin phosphate solutions can be used for the study of thrombin formation with brain extract, they are not suitable with Russell Viper venom because, here, a higher calcium concentration is required at which precipitation of calcium phosphate occurs and prothrombin is removed by adsorption on the former. Therefore, the preparations were dialysed against a solution containing 0·15 M sodium chloride and 0·01 M sodium oxalate for 20 hours at +6° C. The sodium bicarbonate solution was added to give a pH of 7·4.

Fibrinogen solutions were prepared from fresh oxalated human plasma from which prothrombin was removed by barium carbonate or barium sulphate adsorption. Three successive precipitations with 25 p.c. saturated ammonium sulphate were carried out at +6° C. Dialysis and pH adjustments were made as before. Only preparations having a purity of 95–100 p.c. fibrinogen, when compared with the total protein nitrogen, were used.

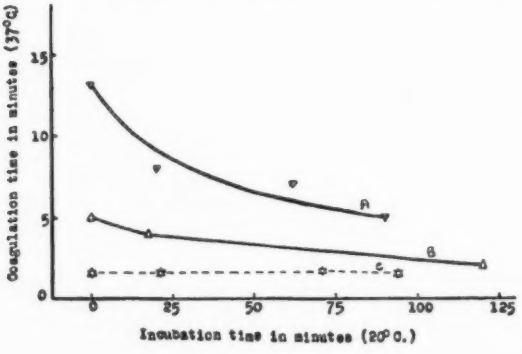
Prothrombin-free plasma (P.F. plasma) was obtained either as filtrate from the prothrombin adsorption, or it was prepared by treating fresh oxalated plasma with one-tenth of its volume of C–γ alumina gel at room temperature for 15–35 minutes.

Prothrombin accelerator was separated from prothrombin-free plasma by fractional precipitation with ammonium sulphate in the cold and the globulin fraction which was salted out at 45 p.c. ammonium sulphate concentration contained the active principle. Dialysis and pH adjustments were carried out as before.

Russell Viper venom was used in the form of the commercial preparation "Stypven". 0·1 mg. was dissolved in 0·5 ml. veronal buffer, pH 7·4. "Cephalin" was prepared either from blood-free calf's brain or from soya bean, according to Eagle's procedure (1935). The ethereal solution was kept in the ice chest and aliquots removed. After evaporating the ether, the residue was suspended in veronal buffer, pH 7·4. Optimum concentration of "cephalin" was determined prior to each experiment. Commercial preparations of egg lecithin were used.

Only colourless or pale yellow preparations of lipids should be used because it is not possible to obtain maximum prothrombin activation with coloured products.

Influence of Russell Viper Venom on Prothrombin.



Mixtures containing 0·4 ml. prothrombin solution, 0·08 mg. Russell Viper venom, 0·8 ml. 0·025 M calcium chloride and 0·8 ml. veronal buffer of pH 7·4 were incubated at 20° C.

0·2 ml. was removed at varying intervals and coagulation observed following the addition of 0·1 ml. fibrinogen solution, or 0·1 ml. of a mixture containing fibrinogen and cephalin. The final fibrinogen concentration was identical in both cases.

Curves A and B (Fig. 1) were obtained when coagulation was carried out with fibrinogen alone whilst curve C indicates the results when fibrinogen and cephalin were used.

From the results, it was calculated that between 7·5 and 12·5 p.c. of prothrombin was converted into thrombin when tested with fibrinogen and 18 p.c. thrombin formation was obtained with fibrinogen-cephalin.

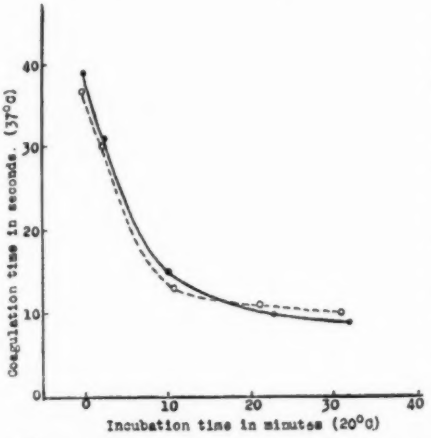


Fig. 2. The prothrombin preparation was obtained from human oxalated plasma which had been stored for 25 days at +16° C.
——·—— denotes that the "cephalin" was isolated from calf's brain.
– – –o– – – denotes that the "cephalin" was isolated from soya bean.

This slight degree of activation is not due to low potency of the venom used because when it was tested on cell-free human oxalated plasma, a clotting time between 50 and 60 seconds was obtained.

The relative ineffectiveness of Russell Viper venom made it necessary to supplement it by addition of lipids. The following mixtures were used: 0·4 ml. prothrombin solution; 0·08 mg. Russell Viper venom in 0·4 ml. veronal buffer, pH 7·4; 0·4 ml. cephalin or lecithin suspension in veronal buffer, pH 7·4; and 0·8 ml. 0·025 M calcium chloride solution. (Incubation temperature was 20° C.)

After desired time intervals, 0·2 ml. of the mixture was removed and 0·1 ml. of a solution added which contained fibrinogen, accelerator plus fibrinogen or prothrombin-free plasma. Coagulation time was measured at 37° C.

A suspension of cephalin from calf's brain could be replaced by a "cephalin" isolated from soya bean without affecting the results, as can be seen from Fig. 2.

The results of experiments using prothrombin prepared from fresh as well as from stored oxalated plasma are summarized in Table 1.

Prothrombins I and II were prepared from fresh plasma, III and IV from plasma which had been stored for 1 week at 20° C., while prothrombins V–VIII and IX–XII had been stored for 4–6 weeks at 20° C. and 7–13 weeks at 6° C. respectively.

Egg lecithin was used as source of lipid with prothrombin I, but for the other instances either calf or soya bean "cephalin" was used.

The results given in Table 1 show that the slight thrombin formation detectable with fibrinogen could be increased markedly by the use of prothrombin-free plasma, or isolated prothrombin accelerator. Further, frequently little difference in activity was seen with the accelerator isolated from human and animal sources (e.g. prothrombin II–IV and VIII, XI and XII). It will also be observed that active prothrombin accelerator can only be obtained from fresh human plasma or from plasma which has been stored for a short time, since the globulin fractions isolated from human plasma stored for 4–10 weeks were comparatively inactive (e.g. prothrombin II, VII, VIII, XI, XII).

The prothrombin-venom-cephalin-calcium mixtures were incubated at room temperature and thrombin formation followed until maximum activity occurred. The end results are given in Table 2.

TABLE 1.

Prothrombin activation by Russell Viper venom and lipid.

Start values (clotting time in seconds).

Assay carried out with	Prothrombin preparation											
	I	II	III	IV	V	VI	VII	VIII	IX	X	XI	XII
Fibrinogen	115	47	87	38	38	150	152	97	66	72	39	42
P.F. human plasma	23	12	13	11	12	25	29	14	—	—	13	14
P.F. rabbit plasma	19	12	14	10	10	22	22	15	22	25	12	12
Globulin fraction + fibrinogen												
From fresh human plasma	*25	*9	—	—	—	—	—	13	14	12	—	—
From aged human plasma	—	48	—	—	—	—	*135	*90	—	—	*40	*44
From fresh rabbit plasma	—	—	—	—	—	—	—	—	15	15	—	—

* Denotes that prothrombin accelerator was prepared from the same plasma as the prothrombin. Globulin fractions isolated from fresh plasma indicate that the plasmas were not kept longer than 8 hours after collection. The globulin fraction from aged plasma used with prothrombin II was isolated from plasma stored for 4 weeks at + 20° C.

TABLE 2.

Prothrombin activation by Russell Viper venom and lipid.

End values (clotting time in seconds).

Prothrombin	Assayed with			Yield of thrombin without accelerator p.c.
	Fibrinogen	Rabbit accelerator	Human accelerator	
II	10·5	—	9	76
III	17·5	14	13	62
IV	9	9	9	100
V	10	11	10	100
VI	23	22	21	90
VII	39	22	22	50
VIII	16·5	15	13	72
IX	17	15	14	82
X	13	14	12	92
XI	9	9·5	8	86
XII	8·5	8·5	8·5	100

The figures given in Table 2 represent the shortest clotting time observed during the incubation period which, when assayed with fibrinogen, varied according to the potency of the prothrombin preparation.

It was found that between 30 and 50 minutes at 20° C. were required to give the recorded results.

When substrates containing optimal amounts of prothrombin accelerator were used, the minimum clotting times were often obtained after incubation for less than one minute.

For the calculation of the yield of thrombin, it was assumed that prothrombin assayed in the presence of optimal amounts of accelerator would give 100 p.c. thrombin.

A standard curve relating thrombin concentration and fibrinogen coagulation time was established (a) by the use of serial dilutions of beef thrombin on oxalated human plasma; and, (b) by serial dilutions of prothrombin preparations with 0·15 M sodium chloride; conversion to thrombin was carried out with brain extract and the thrombin concentration measured after 100 minutes' incubation time.

Conditions in this thrombin standardization were kept as near as possible to experimental conditions. The results obtained from (a) and (b) were found to coincide.

The Effect of Prothrombin Accelerator on the Activation of Prothrombin by Venom-Lipid.

The experimental results given in Table 1 show that prothrombin accelerator is similarly active in the case of venom + lipid as has been found when brain is the source of thromboplastin. However, it has been reported by Fantl and Nance (1948) that dilution of human oxalated plasma with prothrombin-free plasma produced shorter clotting times than dilutions with saline when homologous brain extract was used. In contrast to this phenomenon, no activation was observed in the case of Russell Viper venom + lipid and the different behaviour of the two thromboplastic agents suggested that a quantitative difference exists. In order to establish the relationship between prothrombin accelerator and thrombin formation rate, prothrombin accelerators from different sources and varying concentrations were tested on several prothrombin preparations.

For the assay of prothrombin accelerator, a standard preparation of prothrombin is required. From the results given in Table 3, it will be seen that products giving clotting times between 10 and 15 seconds when tested with optimal amounts of accelerator are suitable. They are readily obtained from 20 ml. stored plasma by barium sulphate adsorption. The elution is carried out with 10 ml. phosphate buffer, pH 7·8, and further treated as outlined earlier. The source of prothrombin accelerator is plasma treated with barium carbonate or barium sulphate suspension. The concentration of the undiluted specimen is designated 100, and dilutions are made with veronal buffer of pH 7·4.

The assay of prothrombin accelerator is carried out in the following manner:

To a series of tubes containing 0·04 ml. prothrombin solution is added 0·05 ml. of the accelerator of varying concentration, followed by 0·08 ml. of a freshly prepared mixture of 0·08 mg. Russell Viper venom in buffer, pH 7·4, and 0·04 ml. cephalin suspension. The tubes are transferred to a water bath at 37° C. and 0·05 ml. of a fibrinogen solution containing 0·5 p.c. fibrinogen is added. Finally 0·08 ml. 0·025 M calcium chloride is introduced and the coagulation time determined. We suggest for the purpose of comparing

potencies to define 100 units of prothrombin accelerator as the smallest amount of active principle which produces the shortest clotting time when assayed under the above conditions.

At least three dilutions of the specimen which indicate suboptimal accelerator activity should be used. The observed coagulation times are plotted against accelerator concentration and from this curve, the activity calculated. Typical results are given in Table 3.

TABLE 3.

Relationship between concentration of prothrombin accelerator and thrombin formation. Activation by Russell Viper venom.

Prothrombin preparation	Source of accelerator	Concentration of accelerator							Units approx.
		100	10	6	1	0·3	0·03	0	
		Clotting time in seconds							
A	Human	9·5	10·5	12	—	28·5	64	67	800
	Rabbit	9	10	11	—	22	55	67	900
B	Human 1	13	13	15	21	—	—	87	1,000
	Human 2	11	12	14	18	—	—	87	900
	Rabbit	14	13	14	18	—	—	87	1,000
	Rat	14	13	15	21	—	—	87	1,000
	Guinea-pig	11	12	13	17	—	—	87	1,200
C	Human	11	11	13	—	—	—	38	1,400
	Rabbit	10	11	13	—	—	—	38	900
D	Human	12	13	—	—	—	—	38	900
	Rabbit	11	14	—	—	—	—	38	500
E	Human 1	14	15	18	24	—	—	100	1,000
	Human 2	15	15	19	23	—	—	100	1,000

Prothrombin A was prepared from two-day-old human oxalated cell-free plasma, prothrombin B, C and E from plasma stored for 10 days at 20° C., prothrombin D was isolated from plasma which had been stored for 4 weeks at 20° C.

From the results given in Table 3, it can be seen, firstly, that the accelerator often shows undiminished activity in the range of 100–10 p.c. plasma concentration; and, secondly, Russell Viper venom + lipid indicates little quantitative difference among the accelerating properties of various species.

It is interesting to compare these results with estimations of the activity of prothrombin accelerator when Russell Viper venom is replaced by homologous brain extract. For this assay procedure a 6 p.c. saline extract of acetone dried human brain powder was heated at 50° C. for fifteen minutes, then by the addition of veronal buffer, the pH was adjusted to 7·4. The suspension was kept for 2 hours at + 6° C. and the milky supernatant fluid used. For 0·04 ml. prothrombin, 0·08 ml. of brain extract and 0·08 ml. of 0·01 M calcium chloride was added. Otherwise the test was carried out as has been done with Russell Viper venom. Results are given in Table 4.

Whilst by the use of snake venom, human and rabbit plasma would appear to contain equal amounts of prothrombin accelerator, the assay using brain extracts indicates markedly lower activity of human plasma.

TABLE 4.

Relationship between concentration of prothrombin accelerator and thrombin formation. Activation by brain thromboplastin.

Prothrombin preparation*	Source of accelerator	Concentration of accelerator									Units approx.
		100	50	25	10	5	3	2	1	0	
		Clotting time in seconds									
A	Human	18	18	22	—	—	—	—	—	67	20
	Rabbit	13	13	13	13	15	—	—	18	67	1,000
B	Human 1	27	29	—	—	—	—	—	—	230	30
	Human 2	27	30	—	—	—	—	—	—	230	30
	Rabbit	18	—	—	20	21	26	30	—	230	800
	Rat	20	24	—	—	—	—	—	—	230	80
	Guinea-pig	20	23	—	—	—	—	—	—	230	80
E	Human 1	24	27	—	—	—	—	—	—	100	15
	Human 2	22	25	—	—	—	—	—	—	100	20
	Rabbit	15	—	—	15	17	—	—	25	100	1,000

* Prothrombin preparations are the same as those given in Table 3.

The identical potency of rabbit oxalated plasma treated with barium sulphate in the two independent assay techniques, makes it a convenient standard.

In order to find out whether barium sulphate adsorbs prothrombin accelerator, the following experiment was carried out. Fresh oxalated cell-free human plasma was treated with sufficient freshly prepared barium sulphate suspension to render it free of prothrombin. From the filtrate, prothrombin accelerator was obtained by fractional ammonium sulphate precipitation. The dialysed product will be referred to as Globulin A. The barium sulphate adsorption was repeated 5 times and the globulin fraction was again prepared from the last filtrate. The dialysed fraction of it will be called Globulin B.

TABLE 5.

Accelerating properties of globulin fraction obtained from oxalated human plasma after barium sulphate adsorption.

Fibrinogen	Prothrombin assayed with			
	Globulin A		Globulin B	
	p.c. 100	p.c. 50	p.c. 100	p.c. 50
69	21	23	22	25

The prothrombin preparation was obtained by elution of the first barium sulphate adsorbate.

Globulin 50 p.c. means dilution 1:1 with veronal buffer, pH 7·4.

The assay procedure was carried out by the use of brain extract because of its greater sensitivity to changes in the concentration of prothrombin accelerator. The clotting times are given in seconds.

The results indicate that the globulin fraction prepared from plasma which had been treated 5 times with barium sulphate showed a very high activity of prothrombin accelerator, and thus it seems that barium sulphate does not remove significant amounts of accelerator from plasma, and behaves as barium carbonate (Fantl and Nance, 1946). The latter finding has been confirmed by Murphy, Ware and Seegers (1947).

DISCUSSION.

The experimental evidence presented suggests that Russell Viper venom cannot adequately convert isolated prothrombin into thrombin but becomes active after combination with lipids present in the cephalin fraction. This assumption is in agreement with observations made on whole plasma by Macfarlane, Trevan and Attwood (1941).

No difference between organ thromboplastins and the venom + lipid system could be detected in the conversion of isolated prothrombin, which suggests a similar mechanism of thrombin formation by both types of thromboplastins. The speed of thrombin formation can be enhanced markedly by prothrombin accelerator when either type of thromboplastin is used.

So far the assay of prothrombin accelerator has been carried out only by the use of organ extracts. Owren (1947) estimates factor V in plasma by testing several plasma dilutions (5–1 p.c.) with a mixture containing prothrombin, fibrinogen, brain-thromboplastin and calcium ions under standard conditions. By the use of dilutions of a standard plasma which is considered to contain 100 p.c. factor V, the concentration of the specimen under examination is found.

Ware and Seegers (1948) suggest for the assay of material containing prothrombin accelerator, a procedure similar to the two stage technique of Warner, Brinkhous and Smith (1936). A standard prothrombin preparation is incubated with a series of dilutions of the material containing the accelerator and from the coagulation times obtained, the concentration is calculated and expressed in units.

According to our experience accelerator prepared from animal plasma was always found to be more active than human material when brain thromboplastin was used. However, frequently no difference in activity was observed in the case of Russell Viper venom.

The divergent results obtained by the two procedures for the assay of prothrombin accelerator allow the following deductions: Russell Viper venom having no particular affinity for any of the mammalian prothrombins and their accelerators will induce thrombin formation according to their concentrations. On the other hand, organ extracts show marked species' differences.

The fact that Russell Viper venom + lipid combination does not differentiate among the various mammalian prothrombin accelerators offers an explanation why prothrombin estimations, carried out by the use of venom, show slight differences in the prothrombin concentration in the blood of a series of mammals, whilst by the use of thromboplastin from animal sources, marked variation in prothrombin activity is found when a one-stage technique is applied.

There is evidence from two other procedures, namely, the two-stage technique of Warner, Brinkhous and Smith (1939), and the barium carbonate adsorption technique of Fantl and Nance (1947b)[2] that only small differences in the actual prothrombin concentration in normal mammalian blood exist. Since the two lastnamed techniques are independent of prothrombin accelerator, it would appear that the weight of evidence is in favour of the assumption that the prothrombin concentration is, within the limit of physiological variation, the same in mammalian blood.

SUMMARY.

Russell Viper venom and calcium ions have little activity as a thromboplastic agent when tested on isolated prothrombin.

Marked activation is obtained following combination with lipids present in the cephalin fraction isolated from animal or vegetable sources.

Prothrombin accelerator has the property of enhancing the speed of thrombin formation when organ extracts or snake venom is used as thromboplastin.

An assay of prothrombin accelerator using venom + lipid indicates nearly equal potency of several mammalian plasmas which is in contrast with results obtained by assay with organ extracts.

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[2] Results will be published shortly.

THE DETERMINATION OF THIAMINE IN WHITE BREAD BY THE THIOCHROME METHOD

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It was Jansen, in 1936, who first measured thiamine by the production from it of the fluorescent oxidation product thiochrome. The oxidation was effected by treatment of thiamine in solution with alkaline potassium ferricyanide. One of the most serious difficulties in the application of the thiochrome method to the determination of thiamine in the tissues and fluids of plants and animals and in foodstuffs is the occurrence therein of substances which fluoresce when excited by ultraviolet light, and of those which, like thiamine itself, are converted to fluorescent substances by the action of alkaline ferricyanide. The former can be eliminated as a source of error by the appropriate use of "blank" estimations in which the ferricyanide is omitted. Substances which become fluorescent only on the addition of ferricyanide, however, present more formidable difficulties. The present paper deals with the discovery of such impurities in extracts of bread and with the analytical procedures adopted for their elimination.

In a preliminary study of the problems involved in the determination of the thiamine content of white bread, it was observed that the *apparent* thiamine present could be increased progressively by increasing the concentration of ferricyanide used to convert the thiamine to thiochrome in a procedure which was essentially that recommended by the Vitamin B_1 Sub-Committee of the Accessory Food Factors Committee of the Medical Research Council and the Lister Institute (1943). This method involves the addition of methanol prior to the addition of alkaline ferricyanide to the thiamine-containing extract; thiochrome is extremely sensitive to the action of excess ferricyanide, and its lability has been found to be very much reduced if methanol is present.

EXPERIMENTAL.

The First Method of Estimation.

The bread was dried and finely powdered. Two samples (5 gm. dry wt.) were shaken up with 105 ml. of acetate buffer, pH 4·0, and a known amount of thiamine was added to one of them to serve as an internal standard. After adding papain and mylase P[1] the mixtures

[1] The sample of mylase manufactured by the Wallerstein Laboratories, New York, was free from thiamine.

were set aside at 35–40° C. to digest for 16–18 hours. The extracts were then centrifuged and filtered. To 2 ml. aliquots of the filtrates were added 2 ml. methanol and 2 ml. 30 p.c. NaOH, followed by 0·25 ml. of potassium ferricyanide solution. After standing for one minute, 25 ml. of isobutanol previously saturated with water were added, and the whole was shaken for one minute and allowed to settle. The solution at this and subsequent stages was protected from strong light.

The supernatant isobutanol solution was clarified by pipetting 15 ml. into 1 ml. of ethanol, and the fluorescence was determined by comparing it with that of a quinine sulphate standard (0·5 μg. quinine sulphate in 10 ml. N/10 H_2SO_4) by means of a photoelectric fluorimeter that was built for the purpose (Stephen, 1948). Hennessy (1941) has stated that a fluorimeter used for the estimation of thiamine should "be of sufficient sensitivity to provide a galvanometer deflection of at least five scale divisions for 0·1 μg. of thiamin chloride".

The instrument used in the present investigation had a sensitivity 15 times this minimum value.

Results.

When this procedure is applied to solutions of pure thiamine, the quantity of ferricyanide employed is not critical; providing there is sufficient present for the conversion of thiamine to thiochrome, further addition of a very considerable excess of ferricyanide will not increase the fluorescence in the isobutanol extract (Table 2). But other substances present in bread extracts which are extracted by isobutanol after oxidation with ferricyanide, and which fluoresce, are very decidedly affected by the concentration of ferricyanide; the degree of fluorescence of the extract increases materially with the further addition of ferricyanide. This point is obvious from the positive correlation between the concentration of ferricyanide and the fluorescence of the isobutanol extract which is illustrated by the data set out in Table 1.

It is apparent from the data presented in Table 2 that the fluorescence due to the thiochrome from pure thiamine is unaffected by the concentration of ferricyanide whether in the presence or in the absence of the complicating factors in extracts prepared from bread, *providing methanol is present.*

TABLE 1.

The relation between the concentration of ferricyanide and the fluorescence developed from a bread extract.

Concentration of K ferricyanide p.c.		Relative fluorescence compared with quinine standard = 50
Series A	2	77·8
	4	80·5
	5	80·9
	6	81·5
	8	81·9
	10	84·0
Series B	5	77·7
	10	81·0
	15	86·7
	20	93·2

TABLE 2.

The relation between the concentration of ferricyanide and the fluorescence developed from a bread extract and from thiamine.

Relative fluorescence compared with quinine standard = 50.

Solution	5 p.c. K ferricyanide	20 p.c. K ferricyanide
A. Bread extract	53·2	69·9
B. Bread extract + thiamine	81·2	97·4
B minus A	28·0	27·5
Pure thiamine solution	45·5	44·7

Rôle of Methanol.

In the absence of methanol, an increase in the concentration of ferricyanide results in the destruction of thiochrome and a loss of fluorescence. There are thus two opposing variables associated with an increase of ferricyanide concentration, viz. a decrease in fluorescence due to destruction of thiochrome and an increase due to impurities in the bread extract. The decrease is observed in the absence of methanol; *the increase is discernible only when methanol is present.*

The possibility that methanol acted merely as a solvent for a fluorescent impurity in the bread extract which, in the absence of methanol, was no longer extracted by the isobutanol solution, was considered and dismissed as a result of experiments, typical data from which are presented in Table 3.

TABLE 3.

Relative fluorescence developed from a bread extract compared with quinine standard = 50.

Concentration of potassium ferricyanide = 20 p.c.

1	methanol added to bread extract before alkali and ferricyanide	70·5
2	no methanol	54·4
3	methanol added at the same time as the isobutanol	53·7

Attempts to Eliminate the Interfering Impurity.

All attempts to differentiate between thiamine and the impurity by selectively destroying or extracting one or the other, either in the bread extract or in the final isobutanol solution, were unsuccessful. A few of these will be mentioned. The fluorescing impurity could not be removed by a preliminary extraction of the bread extracts with isobutanol at pH 3 prior to oxidation, as recommended by the Vitamin B_1 Sub-Committee (1943). When the bread extracts were purified by base exchange on synthetic zeolite (Decalso), the impurity in question accompanied the thiamine. Attempts to destroy the thiamine preferentially by the use of Na sulphite as recommended by Williams, Waterman, Keresztesy and Buchman (1935) failed; both thiamine and the impurity were destroyed by this procedure.

Examination of Flour.

The phenomenon discussed above was observed when the thiamine was extracted from white flour by enzymic digestion, but when acid extraction was employed the fluorescence which developed was not increased by addition of excess of ferricyanide (Table 4).

TABLE 4.

Fluorescence of Flour Extracts.

Relative fluorescence compared with quinine standard = 50.

Method of extraction	2 p.c. K ferricyanide	5 p.c. K ferricyanide	20 p.c. K ferricyanide
Incubation with papain and mylase P	53·2	54·5	69·7
Extraction with 2 p.c. HCl	46·7	46·7	45·9

The quantitative extraction of the thiamine present in flour is effected by acid, but it has been found essential to digest bread with enzymes as during the fermentation of the dough a material part of the thiamine originally present is apparently either phosphorylated or bound in some other way by the yeast cells (Andrews and Nordgren, 1941; Dawson and Martin, 1942).

TABLE 5.

Fluorescence of Bread Extracts.

Relative fluorescence compared with quinine standard = 50 (blank reading subtracted).

	Method of extraction	Concentration of K ferricyanide p.c.		
		2	5	20
1.	Digestion with papain and mylase P	33·0	33·3	49·5
2 and 3.	Digestion with papain and mylase P followed by treatment of the filtrate with HCl			
	(a) Overnight at room temperature at pH 0·47	29·8	32·1	47·4
	(b) Heated 5 minutes at pH 0·34	—	34·0	49·3
4.	Heated with 0·02 N HCl 10 minutes and then digested with papain and mylase P overnight	31·5	34·2	56·5
5.	Heated with 0·02 N HCl 10 minutes	22·0	22·6	23·3
6.	Extracted with 2 p.c. HCl overnight at room temperature	21·4	20·7	20·3
7.	Filtrate from 6 incubated overnight with mylase P	29·5	28·6	29·3
8.	Further incubation of 7 with papain added	29·8	30·2	31·8*

* This small increase in the reading with 20 p.c. K ferricyanide is due to the papain. It occurs when papain is incubated with pure thiamine solution (no bread present).

Modified Method of Extracting Thiamine from Bread.

The impurity in the bread extracts was not destroyed by treatment with acid after incubation, or by the action of acid on the bread itself before enzymic digestion (Table 5, Nos. 2, 3 and 4), but it was observed that a satisfactory separation of thiamine from bread could be effected by acid extraction of the bread itself, followed by treatment of the filtered extract with the enzyme preparation mylase P after adjusting the reaction to pH 4 (Table 5, No. 7). Under these conditions, the material responsible for the interfering fluorescence is either not produced or not extracted.

Purification of Bread Extracts by Base Exchange.

Acid extraction followed by enzymic digestion of the filtrate was employed for a series of determinations of thiamine in bread. The data in Table 6 are typical of the findings. In Part A the extract was used direct; in Part B it was purified by base exchange on Decalso. This purification markedly reduced the blank reading. It also invariably reduced the fluorescence developed by ferricyanide from the bread extract alone, while at the same time the difference between the readings of the extracts with and without added thiamine was increased (Table 6). It is considered that this increase was due to the removal of impurities which reduced fluorescence by absorbing part either of the exciting or of the fluorescent light. It is probable that the reduced fluorescence developed from the bread extract itself may be traced, not to loss of thiamine during the base exchange, but to the removal of impurities which fluoresced on the addition of alkaline ferricyanide. For this reason, in the method that was adopted for the estimation of thiamine the bread extracts were purified by passing them through columns packed with Decalso.

TABLE 6.

Fluorescence of bread extracts.

Relative fluorescence compared with quinine standard = 50. (Mean of 5 breads.)

Reading of bread extract minus blank reading			Reading due to added thiamine (extract plus thiamine minus extract alone)		
2 p.c. K ferricyanide	5 p.c. K ferricyanide	20 p.c. K ferricyanide	2 p.c. K ferricyanide	5 p.c. K ferricyanide	20 p.c. K ferricyanide
	A. Extract not purified by base exchange				
28·3	28·0	26·9	28·3	27·7	27·9
	B. After base exchange on Decalso				
26·0	26·5	27·2	30·5	29·9	28·9

PROCEDURE FINALLY ADOPTED FOR THE DETERMINATION OF THIAMINE IN WHITE BREAD.

Extraction of Thiamine.

The bread was allowed to stand for one, or occasionally two days after baking. It was then cut into thin slices, dried in a current of air at tempera-

tures not exceeding 45° C. and finely ground. Two 5 gm. samples of bread thus dried were taken for each estimation. To one, 101 ml. 2 p.c. HCl (W/V) were added, to the other 100 ml. 2 p.c. HCl + 1 ml. thiamine solution containing approximately 1 mg. thiamine HCl[2] in 100 ml. 2 p.c. HCl. After standing for 24 hours at room temperature with occasional shaking, the extracts were separated from the solid residues by centrifuging and filtering through extracted filter paper. To 50 ml. aliquots of the filtrates were added 15 ml. acetate buffer, sufficient 10 p.c. NaOH to bring the reaction to pH 4·0–4·4 and 0·2 gm. mylase P, and the whole was incubated for 18 hours at approximately 40° C. and filtered.

Purification of Extracts by Base Exchange.

For this purpose, use was made of the synthetic zeolite Decalso activated in bulk according to the method of Hennessy (1941). For each assay a column 8 × 0·7 cm. was prepared by the method of Brown, Hamm and Harrison (1943). Aliquots of the bread extracts (25 ml.) were passed over the columns which were then washed three times with 5 ml. H_2O. The thiamine was removed with 25 p.c. KCl in N/10 HCl (10 ml. + 10 ml. + 5 ml.). It is advisable to select tubes for the base exchange which require about 20 minutes or longer for the passage of 25 ml. liquid—if the flow is too rapid a loss of 2 to 3 p.c. of the thiamine may result.

Measurement of Thiamine in Purified Extracts.

Method A (in the presence of methanol). To 3 ml. aliquots of the effluents from the base exchange were added 3 ml. methanol, 2 ml. 30 p.c. NaOH and 0·1 ml. 5 p.c. potassium ferricyanide solution, which was the amount required to give a yellow colour perceptible for one minute. After standing for one minute, 25 ml. isobutanol saturated with water were added and the mixture was shaken for one minute and allowed to settle in the dark; 15 ml. of the supernatant liquid were pipetted into 1 ml. ethanol and 10 ml. of this solution were transferred to a cuvette. The fluorescence was compared with that of 10 ml. of the standard quinine solution containing 0·5 μg. quinine sulphate. The standard quinine solution was renewed after every measurement (Clausen and Brown, 1944). The development and measurement of thiochrome in aliquots of the effluents were always carried out in duplicate or triplicate. Blank determinations (without ferricyanide) were made. The fluorimeter was so constructed that the fluorescent solution was exposed to the U.V. light only during the few seconds required to make a reading. Several readings of each were taken and averaged, after allowing for the small loss of fluorescence occurring during exposure to the exciting light. This loss was approximately 0·3 to 0·4 p.c. in 5 seconds.

[2] The pure thiamine HCl lost 4·2 p.c. of its weight *in vacuo* over P_2O_5.

Method B (*without methanol*). Watson (1946), in a study of factors influencing the oxidation of thiamine to thiochrome in the absence of methanol, concluded that the most satisfactory procedure was to add the thiamine solution to the previously mixed potassium ferricyanide and strong NaOH.

To 3 ml. of 30 p.c. NaOH were added 0·05 ml. 0·5 p.c. potassium ferricyanide and, with shaking, 3 ml. bread effluent. The amount of ferricyanide was sufficient to give a yellow colour perceptible for 1 minute. After standing for 1 minute, 25 ml. isobutanol saturated with water were added and the mixture was shaken for 1 minute and centrifuged; 15 ml. of the supernatant layer were added to 1 ml. ethanol, and the fluorescence was measured as in Method A.

The measurement of thiamine in the purified extracts when carried out by the two procedures A and B gave results which agreed to within 1 p.c.

Calculation of Thiamine in Bread.

The bread sample to which thiamine was added before extraction provided an internal thiamine standard. By a comparison of the fluorescence due to the bread alone with that of the bread to which a known amount of thiamine had been added, the amount of thiamine in the bread itself could be calculated.

As a check on the recovery of thiamine, a comparison was made of the thiamine concentration in effluents of bread to which thiamine had been added before extraction with that in effluents to which an equivalent amount of thiamine in acid KCl was added direct. It was assumed that the thiamine originally present in the bread and that added before extraction were evenly distributed throughout the liquid extract, but were not present in the solid phase. In a series of tests it was found that the recovery of thiamine added at the beginning averaged 97·0 p.c. as compared with that of thiamine added to the effluent. The use of an internal standard makes allowance for this loss.

DISCUSSION.

While attempting to determine the amount of ferricyanide to be added to extracts of bread for the oxidation of thiamine, it was discovered that the fluorescence produced increased progressively with increasing concentrations of ferricyanide. No satisfactory criterion could be found for deciding when the correct amount of ferricyanide had been added.

This phenomenon was observed only in the presence of methanol. When the oxidation of thiamine was carried out without methanol, the destructive action of excess ferricyanide on thiochrome, which is inhibited by methanol, obscured the rise in fluorescence due to an impurity in the bread extracts. But no confidence was felt in the results obtained under these circumstances, since it was apparent that the actual fluorescence was the resultant of several variables of which the concentration of thiamine was only one.

Before a satisfactory determination of thiamine in bread could be achieved, it was necessary that the interfering impurity should be eliminated.

It was observed that flour exhibited the same behaviour as bread when the thiamine was extracted by the action of enzymes, but not when acid extraction

was used. Following on this observation, a satisfactory method of extracting thiamine from bread was arrived at. Thiamine and its compounds were extracted from bread by acid, and a subsequent enzymic digestion of the filtered extract converted all the thiamine compounds present to free thiamine. The offending impurity was either not formed or not extracted.

Further interfering impurities were eliminated by passing the bread extracts over columns of Decalso. This step proved to be essential for an accurate determination of thiamine in bread.

SUMMARY.

When the thiamine in white bread is measured by a modification of the thiochrome method in which methanol is present, the apparent thiamine content can be increased progressively by increasing the concentration of ferricyanide used for oxidation.

When thiamine is extracted from flour by enzymic digestion, a similar phenomenon is observed, but not when acid extraction is employed.

By extracting bread with acid at room temperature and digesting the filtrate with an enzyme, a satisfactory extraction of thiamine can be achieved.

It is necessary to purify bread extracts by base exchange.

The method of estimation finally adopted is described. Two modifications of the oxidation procedure, with and without methanol respectively, gave similar results.

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THE THIAMINE CONTENT OF WHITE BREAD IN ADELAIDE

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A brief survey has been made of the thiamine content of white bread in Adelaide. Between September, 1947, and May, 1948, 54 loaves of white bread were obtained from 31 bakeries in and around the City of Adelaide. The bread was all ordinary white bread. Special breads, such as those baked with extra gluten, were not included in the survey. The loaves were all of the type known as sandwich loaves.

The thiamine content of the breads was measured by the method described in the preceding communication (Dawbarn, 1949). The moisture contents of the fresh breads and of the air dried breads used for analysis were determined, and the results have been expressed in terms of μg. thiamine hydrochloride per pound of fresh bread. The thiamine content was found to vary from 330 to 690 μg. thiamine hydrochloride per pound of bread as purchased (one day after baking). The mean value was 470, S.E. = 10. The frequency distribution of breads according to the thiamine content is shown in Fig. 1.

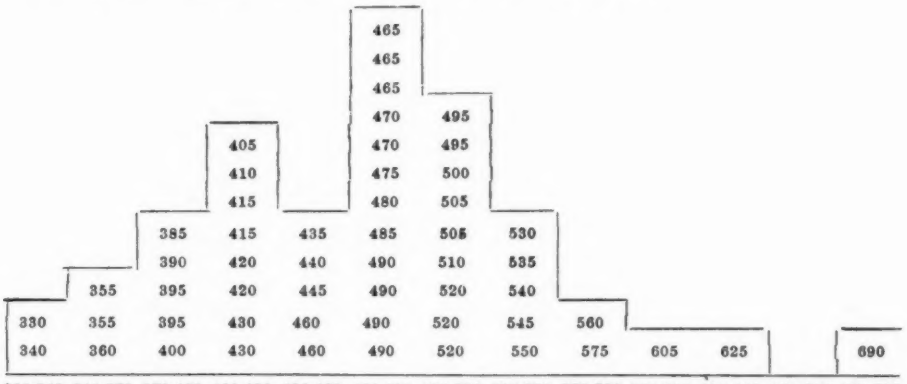

Fig. 1. Frequency distribution of breads according to the content of thiamine. Class interval 30 μg. thiamine hydrochloride per pound.

It has been reported that the mean value of the vitamin B_1 content of Australian white flour lies between 1·56 and 1·79 μg. per gm. (Nutrition Committee, National Health and Medical Research Council, 1941). These figures may be compared with the vitamin B_1 content of bread as determined in the present investigation if the values for bread are calculated to a moisture content of 12 p.c. So expressed, the vitamin B_1 content of bread becomes 1·12–2·34, mean 1·59 μg. per gm.

A more comprehensive survey than the present would be required to determine with certainty the mean thiamine content of the white bread consumed in Adelaide. But certain tentative conclusions may be drawn from these data.

The average consumption of white bread per adult male in Adelaide was found to be 0·59 pound per day (Nutrition Committee, National Health and Medical Research Council, 1945). If the mean thiamine content were 470 μg. per pound, the thiamine supplied from white bread would be 277 μg. per day, which is 18 p.c. of the recommended daily allowance for a moderately active man weighing 70 kg. (Food and Nutrition Board, National Research Council, Washington, 1945).

SUMMARY.

A survey has been made of the thiamine content of white bread in Adelaide. This was found to vary from 330 to 690 μg. thiamine hydrochloride per pound, mean 470, S.E. = 10.

It is estimated that the white bread consumed would supply 18 p.c. of the recommended allowance for thiamine.

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INACTIVATION OF THE RECEPTOR-DESTROYING ENZYME OF *V. CHOLERAE* BY IMMUNE SERA

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In the course of work with the receptor destroying enzyme (RDE) of *V. cholerae* it became desirable to have available a corresponding immune serum. Rabbits were therefore inoculated with active filtrates by the intravenous route for various periods. No trace of anti-RDE effect could be demonstrated in their sera although high titre vibrio agglutinins and a potent anti-mucinase effect (described in another paper) were obtained. Equally negative results were obtained in mice and guinea-pigs.

An attempt was therefore made to produce an immune serum by the use of adjuvants and subcutaneous inoculation. This was successful in providing active antisera, the properties of which are described in this paper.

It will be shown that all the characteristic activities of RDE can be prevented by the antiserum including its destruction of virus receptors, the uncovering of "T" antigen in red-cells, the liberation of fixed virus from the mouse lung *in vitro* and the destruction of the Francis inhibitor in serum or mucin solutions.

EXPERIMENTAL.

Materials.

RDE. The types of preparation used, crude extract heated at 55° C. in the presence of Ca excess, and enzyme purified by adsorption to and elution from fowl red cells, and the standard method for titration of receptor destroying power on red cells are described by Burnet and Stone (1947).

Immunization procedure. A high titre RDE eluate prepared from heated material containing no appreciable mucinase was used. Equal volumes of eluate, liquid paraffin, and a locally manufactured "lanoline base" approximately equivalent to the preparation Falba used by American authors, were emulsified in a mortar to a stable creamy emulsion. Approximately 2 ml. of the mixture were inoculated subcutaneously into each of two rabbits R228 and 229. A second similar injection was given two weeks later and the rabbits bled in a further two weeks. Sera were stored in the cold with the addition of 1:10,000 methiolate.

The anti-mucinase serum R148.3 used in some of the experiments has been described previously (Burnet, 1948a).

Virus inhibitor titrations. These were carried out by the technique described by Burnet (1948b). Briefly serial dilutions of the material to be tested were mixed with five agglu-

[1] This work was carried out under a grant from the National Health and Medical Research Council, Canberra.

tinating doses of heated LEE virus; after 30 minutes suitable fowl cells were added and the inhibitor dilution showing partial agglutination taken as the endpoint.

The Titration of Anti-RDE.

Titration can be carried out by several methods, the simplest being derived directly from the standard method of RDE titration previously described (Burnet and Stone, 1947). A suitable dilution of standard RDE corresponding to five effective doses is prepared and this is used as diluting fluid for the preparation of serial dilutions of the serum to be tested. The dilutions so prepared are placed in a waterbath at 37° C. for 30 minutes. An equal volume of 1 p.c. fowl erythrocytes is added and after a further 30 minutes in the waterbath to allow the action of unneutralized RDE on the red cells one drop of active virus of influenza A Melbourne diluted to contain 100 agglutinating doses is added. The tubes are shaken and replaced in the bath for 30 minutes. Readings of the pattern of deposited cells are then made.

Table 1 shows the results obtained by this method using in addition three other strengths of standard RDE.

TABLE 1.

Titration of anti-RDE Serum 228–3.

Dilution of RDE	Dilutions of serum 228–3									Control	End-point
	10	20	40	80	160	320	640	1,280	2,560		
1,280 (2·5 units)	—	+	++	++	++	++	++	+	—	—	1,280
640 (5 units)	±	±	+	++	++	+	—	—	—	—	320
160 (10 units)	±	+	+	++	±	—	—	—	—	—	120
80 (20 units)	±	+	++	±±	—	—	—	—	—	—	100

++ + ± degrees of agglutination.

The absent or incomplete agglutination in the first two columns is due to the nonspecific inhibition of haemagglutination by strain Melbourne by low dilutions of serum. On this test the serum titre would be taken as 320 allowing for some irregularity in the results; the serum neutralizes approximately 0·5 to 1 times its volume of a typical high titre RDE filtrate.

It should be noted that unless the serum is highly active in its anti-RDE capacity, complications will arise by the agglutination (T agglutination) of cells acted on by RDE in the lower dilutions of serum. In all earlier experiments including that shown in Table 1 the serum was first absorbed with RDE treated cells. If high titre sera such as 228·3 are tested with 5 units of RDE the endpoint is beyond that of the T agglutinin titre of the serum and they can therefore be used unabsorbed.

The second method of titration is to make use of the T agglutinin effect (Burnet and McCrea, 1946; Lind and MacArthur, 1947). One way is to carry out the experiment as in the first method but instead of adding virus as indicator of change to add one drop of undiluted normal rabbit serum. This gives a final dilution of 1:20 and agglutinates cells changed by RDE but not normal cells. The readings give the same endpoint but the cells agglutinated are of course on opposite sides of the endpoint.

Another method is to make serial dilutions of the enzyme in a diluting fluid of the serum to be tested at a 1:20 concentration in saline. After 30 minutes at 37° C. an equal volume of 1 p.c. human cells is added to each tube and the racks replaced in the waterbath at 37° C. for action of unneutralized RDE. They are then shaken and allowed to sediment in the cold. Agglutination occurs in all tubes in which there is unneutral-

ized RDE. In a typical experiment sera 228–1 (normal) and 228–3 (immunized) gave endpoints with dilutions of RDE of 1,280 and 10 respectively. A control series of RDE dilutions in saline was treated similarly and after removal from the waterbath a drop of undiluted normal rabbit serum was added to produce indicator agglutination. The titre obtained was 3,200. It is evident therefore that the normal serum had a slight inhibitory effect on the enzyme, reducing its apparent titre from 3,200 to 1,280, and the immune one a very marked effect. In this experiment serum 1:20 neutralized approximately twice its volume of enzyme of standard titre.

In all experiments on the point it has been found that a higher titre is obtained for the anti-RDE effect if serum and enzyme are allowed to react for 30 minutes before the red cells are added than if this is done immediately.

The necessity for an adequate time of action before testing is shown in the following experiment using T agglutination as the indicator.

Serial dilutions of the sera concerned were made in a diluting fluid containing 1:500 of an active eluate of titre 3,200 and 1:250 of normal rabbit serum in buffered saline. Three series were made respectively two hours, thirty minutes and one minute before an equal volume of 1 p.c. human RBC's was added, the mixtures remaining over the first two periods at 37° C. in a waterbath. After the final addition of red cells the tubes were kept at 37° C. for 30 minutes and then removed from the bath. One drop of undiluted normal rabbit serum was placed in each tube and the racks shaken and left in a refrigerator for an hour for agglutination to occur (Table 2).

TABLE 2.

Influence of time of contact on titre of anti-RDE activity.

Serum	Time of Contact	Titre
228–1 Normal	2 hours	<10
228–3 Anti-RDE	2 hours	350
228–3 Anti-RDE	30 minutes	300
228–3 Anti-RDE	1 minute	80

TABLE 3.

Ring tests with RDE and antisera.

Serum	Crude (1,600)	Eluate A (4,000)	Eluate D (2,500)	Boiled crude Broth	
228–1 Normal	—	—	—	—	—
228–3 Anti-RDE	++	++	++	±	—
229–3 Anti-RDE	++	+	+	+	
148–3 Antimucinase	+	—	—	+	—

Readings made after 3 hours at room temperature.

Precipitin Reactions of Antiserum and Enzyme

When anti-RDE serum is used in ring tests with appropriate material containing a high concentration of RDE (1,000 units) a well defined precipitin reaction can be observed. This is not produced by the anti-mucinase (and anti-

vibrio) serum 148·2. Table 3 combines tests made throughout with undiluted reagents including normal, anti-RDE and anti-mucinase sera, crude agar filtrate and two red-cell eluates. In all tests undiluted serum was run in carefully below the antigen solution to give sharply defined layering of the reagents.

Inhibition of the Action of RDE in Destroying Francis Inhibitor.

It has recently been shown that the serum inhibitor of haemagglutination by heated LEE virus (Francis, 1947) is destroyed by RDE (Anderson, 1948). Similar findings have been obtained with glandular mucin from various sources (Burnet, 1948b). The activity of anti-RDE serum has therefore been tested using this action of RDE as indication of its effect.

In the experiment to be described a sample of uterine cervical mucin was used primarily because it was the only one available which had a high content of blood group substance A. Various mixtures as shown in Table 4 all contained the following final dilutions:

Mucin 1:10 (of a stock solution) enzyme 1:40 serum 1:20. Enzyme and serum were allowed to react for thirty minutes at 37° C. and the mucin solution then added. The tubes were returned to the waterbath at 37° C. for one hour and the contents then tested (a) for the physical character of the mucin by ACRA test (Burnet, 1947), (b) for titre of virus inhibitor and (c) for power to block the agglutination of human A cells by an anti-A serum.

TABLE 4.

Influence of antisera on the actions of vibrio enzymes on human mucin.

Mucin treated with	ACRA	Francis Inhibitor	Anti-B.G.A.
RDE Normal serum	+	<10	2,000
Anti-RDE 228	+	1,280	2,000
Antimucinase 148	+	<10	2,000
RDE-M Normal serum	—	<10	2,000
Anti-RDE 228	±	1,280	2,000
Antimucinase 148	+	<10	2,000
Normal serum alone	+	1,280	2,000

RDE Vibrio filtrate treated at 55° C. to destroy mucinase.

RDE-M Untreated filtrate containing both mucinase and RDE.

Table 4 shows the complete inhibition of the destructive action of RDE on Francis inhibitor by the anti-RDE serum 228. With the relatively large serum concentration used there is a slight anti-mucinase effect but other experiments have shown that this is less than 1/32 of the anti-mucinase activity of serum 148. It will be noticed that the vibrio enzymes have no effect whatever on the blood group substance activity and that the titre of virus inhibitor is independent of the physical state of the mucin.

Other Methods of Demonstrating Action of Anti-RDE Serum.

The immune sera described in this paper have been used extensively by other workers in this laboratory as an additional means of confirming that a certain biological activity

was actually due to the enzyme. Fazekas de St.Groth (1948) has shown that the addition of anti-RDE serum to RDE preparations removes their activity in liberating LEE virus either living or heated from the mouse lung *in vitro*, and that the neutralizing activity of the serum was approximately the same as that determined by red-cell methods. The anti-mucinase serum 148–3 had no such effect. Stone (1948) has shown that the protective effect of RDE action against subsequent infection with influenza viruses in chick embryos and mice is also eliminated by the antiserum.

In work to be published shortly Burnet (1949) has found that the action of RDE on ovomucin can be demonstrated without the use of a biological indicator, by testing the precipitability of ovomucin solutions with protamine. Under suitable conditions RDE destroys this precipitability; the reaction is prevented by serum 229–3 (anti-RDE). The somewhat similar action of vibrio mucinase is uninfluenced by anti-RDE serum.

DISCUSSION.

The anti-RDE sera described in this paper have proved of much value in all the recent work from this laboratory which has been concerned with the relation between RDE on the one hand and the enzyme associated with viruses of the mumps-influenza group. Since crude cholera vibrio filtrates obviously contain a variety of enzymes it has always been necessary to establish that the various activities we have ascribed to RDE are in fact due to a single molecular species. At least one other enzyme which we refer to as vibrio mucinase, present in cholera vibrio filtrates, acts on the same substrates (various mucins) as RDE. It was of considerable interest and from our point of view fortunate that first attempts to produce anti-RDE by immunization with crude high titre preparations gave no anti-RDE whatever though a potent anti-mucinase. The anti-RDE serum was eventually prepared by immunizing with semipurified enzyme emulsified in paraffin oil and found to contain only doubtful traces of anti-mucinase. It is therefore highly unlikely that the anti-RDE sera contained significant amounts of antibody against any other enzyme produced by the cholera vibrio.

We now regard a biological activity of a *V. cholerae* filtrate as due to the activity of RDE if (1) it is shown both by preparations heated to 55° C. in the presence of excess Ca, and by red-cell eluates, to an extent proportional to the RDE titre obtained by the standard method and (2) the activity is neutralized by anti-RDE serum and not by the serum 148–3 which contains anti-mucinase and a variety of anti-vibrio antibodies but no anti-RDE.

Our whole interest in anti-RDE serum has been as a reagent for the study of the biological activities of the enzyme and no extensive study of the conditions for its production or the character of the antigen-antibody reaction has been made. There is nothing to indicate that the antibody differs importantly from other anti-enzymes that have been studied. Its significance is merely to strengthen our claim that the following activities are functions of a single enzyme "RDE."

(1) The destruction of virus receptors on red-cells;

(2) the exposure of T-agglutinogen on red-cells;

(3) the destruction of virus-inhibitor in serum and in various types of mucin solution;

(4) the liberation of adsorbed influenza virus (living or heated) from the respiratory surface of the excised mouse-lung;

(5) the prevention of infection in the allantoic cavity or in the mouse lung by influenza viruses;

(6) the destruction of the precipitability of ovomucin by protamine.

SUMMARY.

Using a preparation purified by elution from red-cells an active antiserum against the vibrio enzyme RDE has been prepared by immunization of rabbits with the antigen emulsified in paraffin oil with a lanoline base.

The antiserum precipitates with enzyme preparations of sufficient titre and is capable of neutralizing all the biological activities of the enzyme.

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HISTOLOGICAL INVESTIGATION OF GLYCOGEN IN SKIN AND HAIR

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Following Claude Bernard's discovery in 1857 of glycogen in the tissues, the glycogen content of the skin of mammals was repeatedly examined. However, only histological methods were employed. Best's carmine stain and the iodine stain were used to demonstrate the presence of glycogen in skin sections. With the aid of these methods, it was found that glycogen occurs in the epidermis of human embryos in the first half of intra-uterine life and then disappears. In the second half of intra-uterine life, glycogen begins to appear in the outer root sheath of the hair follicle and may be found there throughout life according to histological evidence. No great significance has been attached to these observations by recent investigators and, as far as we know, no further work has been published since 1921 (Sasakawa).

Schultz and Schmidt (1931) slightly altered Best's carmine method for the detection of glycogen and claimed that the carminophily observed with the new formula indicated crystals, colloidal droplets and sphaeroliths of uric acid. In view of the occurrence of large amounts of uric acid in the keratinous integument (Bolliger and Hardy, 1945), this and another method of Schultz and Schmidt were also investigated.

Hotchkiss (1948) described a method for the staining of a wide variety of natural polysaccharides, which are oxidized under mild conditions with periodic acid. Polyaldehydes are formed and these yield coloured compounds with Schiff's reagent, fuchsin-sulphite. Polysaccharides react to a varying degree but a vigorous reaction was claimed for glycogen.

Recently, however, it has been shown that the extradermal hair contains unexpectedly large amounts of glycogen (300–500 mg. p.c.) (Bolliger and McDonald, 1948) as determined by chemical methods. Consequently, histological methods which might indicate the origin and local distribution of this polysaccharide were applied to skin and hair.

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METHODS.

Pieces of skin of man, sheep, rabbit and phalanger (*Trichosurus vulpecula*) were hardened in formalin or alcohol and paraffin sections (tangential) were prepared in the usual way and stained by three methods. Paraffin sections of human hair, sheep fleece and rabbit and phalanger fur were also prepared and stained similarly.

Best's Carmine Stain.

This method has been fully described in text books of histology and standard procedure was adopted (Carleton, 1938).

Schultz' and Schmidt's Carmine Stain.

In the formula of Schultz and Schmidt (1931) lithium carbonate is used in place of potassium carbonate in Best's method; otherwise, the two stains are identical. According to Schultz and Schmidt, who only dealt with renal tissue, cytoplasm and connective tissue stain slightly red; glycogen remains unstained if the correct staining time (5 minutes or less) is adhered to and colloidal droplets and sphaeroliths of uric acid stain bright red while sodium biurate remains unstained.

Hotchkiss' Periodic Acid-Fuchsin Sulphite Stain.

The technique as recently described by Hotchkiss was applied to the sections.

RESULTS.

Skin Sections.

Best's Carmine Stain.

Skin sections of the phalanger, sheep, rabbit and man showed globules, which stained brilliantly red, located in the lower half of the outer root sheath of the hair follicle up to somewhere about the level where the sebaceous glands connect with the hair follicle. By their depth of staining, these carminophilic globules were definitely distinguished from nuclei, cytoplasm and connective tissues which stained pink. In the rabbit and in the phalanger, in addition, the hair root near the papilla frequently stained red and sometimes contained red globules similar to those seen in the outer root sheath. However, in the rabbit, the granules in the root sheath were fewer in number and smaller in size than in the other cases examined (Fig. 1).

Schultz' and Schmidt's Carmine Stain.

The carmine stain of Schultz and Schmidt gave the same results as Best's carmine and according to their claims, would identify the granules as uric acid. This assumption was substantiated by the present authors to some extent inasmuch as uric acid, urate precipitates in urine and doubtful uratic concrements in kidney sections and skin sections of gout patients sometimes were found to stain red with carmine.

In view of there being two or more possible interpretations for the carminophilic granules present in the outer root sheath, a search was made for tests which would indicate the chemical nature of these deep-red-staining elements in the outer hair root sheath.

Extraction with boiling water: Small pieces of sheep skin were boiled in water for about 10 minutes prior to sectioning. After staining with carmine, although the general histology still appeared to be normal, no brilliant red globules were noted. In some cases, however, the location of the red globules previously present in the outer root sheath was

Fig. 1. Microphotograph of a rabbit skin section stained with carmine × 200. Deeply staining granules are seen in the outer root sheath of the hair follicles.

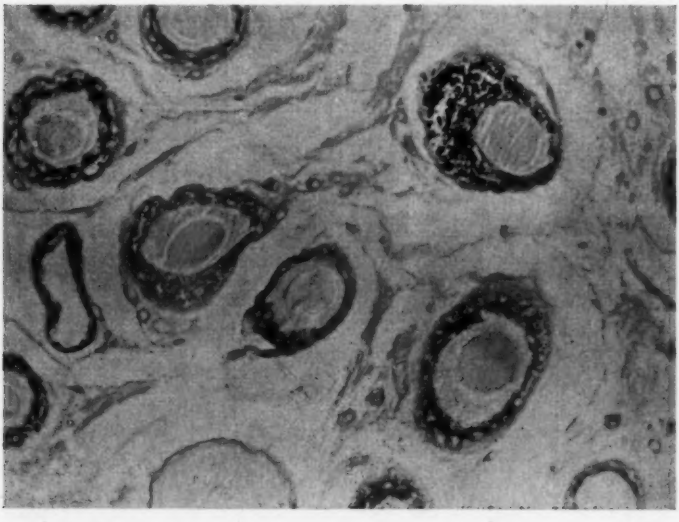

Fig. 2. Microphotograph of a sheep skin section stained with periodic acid fuchsin sulphite. × 200. The section was cut approximately parallel to the surface just below the sebaceous glands. Deep staining globules are again clearly visible in the outer root sheath of the hair follicles.

definitely recognized by the presence of a globular stroma very faintly stained or not stained at all.

Solubility in water containing piperazine: A skin section (I) from each experimental animal was placed for five minutes in 2–4 p.c. cold aqueous piperazine solution, a second section (II) was placed for five minutes in cold distilled water, while a third (III) was left untreated. When these three sections were stained with carmine according to Best or Schultz and Schmidt, section III showed the usual picture, section I showed that most of the carmine staining globules had been dissolved out and section II showed that only a negligible amount of carminophilic material had disappeared.

This experiment indicated that the carminophilic material is more soluble in piperazine solution than in water.

Schultz and Schmidt also devised a methylene blue-picrate stain for sodium biurate, to be used in conjunction with the carmine stain. The procedure was claimed to give sodium biurate present in sections a brilliant green colour. The technique as described by the authors was applied to the skin sections of the experimental animals and was found to stain the inner root sheath of the hair follicle a brilliant green. In all probability, however, this reaction is not indicative of the presence of uric acid or glycogen because it was still present after boiling out the sections or treating them with piperazine solution.

Treatment with saliva: When the skin sections of the sheep and the rabbit were treated for one hour with cold saliva (amylase) before staining, in both techniques the red granules in the outer root sheath disappeared, while, on the other hand, some parts of the hair shafts near the papilla which had previously stained red, retained their colour.

Iodine reactions: Skin sections of the sheep and rabbit when stained with Lugol's iodine solution showed deep brown staining material in the outer root sheath. The hair shaft itself stained yellow. The dark staining material was not observed after treating the slide with saliva before staining. This stain, however, was found to fade very rapidly which made successful observation difficult.

Periodic Acid-Fuchsin Sulphite Stain.

As with Best's carmine stain identical granules, now stained a deep purple (Fig. 2), appeared in the lower half of the outer root sheath of man, sheep, rabbit and phalanger when treated with the periodic acid-fuchsin sulphite stain. However, the fine granules in the root sheath of the thin hairs of the rabbit and man, which still showed up with the carmine stain, were difficult to observe. When the periodic acid-fuchsin sulphite technique was applied to sections which had previously been treated with carmine, these fine granules in the outer root sheath of the small hairs were no longer observed, although the root sheath itself assumed a deeper purple colouration. The large granules, previously stained red, now exhibited a purple colour. When the treatment with periodic acid was omitted, no staining with fuchsin sulphite was observed in any part of the sections.

Intact Hair and Hair Sections.

Best's Carmine Stain.

Only prolonged exposure overnight of sectioned hairs to the carmine solution brought about staining of the medulla of rabbit hair. No staining was observed in the cortex or with whole hairs.

Periodic Acid Fuchsin Sulphite Stain.

Intact Hair: The intact hair stained only weakly. The majority of the cut ends of medullated hairs, however, showed a deep purple staining where the fuchsin sulphite stain penetrated into the medulla for a distance of two to ten cells.

Sectioned hairs: The same staining procedure employed for skin sections produced a deep purple colouration in the cells of the medulla of the hair of the rabbit and phalanger. In addition to a diffuse stain, very deeply coloured granules were seen.

Extraction of Hair with Boiling Water.

On boiling out rabbit fur, glycogen is obtained as pointed out previously. This glycogen, isolated by chemical processes stained purple with periodic acid-fuchsin sulphite. The medulla of rabbit hair also stained purple by the method of Hotchkiss, but rabbit fur which had been boiled out was still stained by Schiff's reagent, though somewhat less intensely.

DISCUSSION.

In spite of other skin tissues being coloured pink, Best's carmine technique clearly delineates granules which stain intensely red in the outer hair root sheath of the four species examined. These structures are not observed with the ordinary histological stains and the precise nature of the staining reaction is obscure.

Practically similar results are obtained with the fuchsin sulphite method following oxidation by periodic acid (Hotchkiss, 1948) and, in addition, the chemistry of the staining reaction is better understood.

The methods of Schultz and Schmidt were of little use in the present investigation, except for bringing out the fact that the carminophilic granules in the outer hair root sheath are considerably more soluble in piperazine solution than in water and that, in this respect, they somewhat resemble uric acid, which is readily soluble in aqueous piperazine and almost completely insoluble in cold water. Therefore, the hypothesis that the sparingly soluble carminophilic granules may represent a combination of uric acid with glycogen is not entirely unfounded. It could be assumed that the uric acid is dissolved out from this combination by the specific uric acid solvent piperazine, leaving behind the glycogen component, which now becomes readily water soluble. It has to be pointed out that in the past the red granules in the root sheath were considered to be glycogen simply on the basis of their exhibiting a similar staining reaction to that observed for glycogen in other tissues such as liver, in spite of the fact that they are much less soluble and much more stable than the glycogen encountered in other organs.

Additional proof of the polysaccharide nature of the carminophilic granules is supplied by the periodic acid-fuchsin sulphite method which stains the same structures deep purple. The surrounding skin tissue, the nuclei being the main exception, takes on a light purple colour but with the omission of periodic acid treatment, the sections including the root sheath, are not stained by fuchsin sulphite. This could be interpreted as indicating that compounds with adjacent glycol groupings are present in most parts of the skin but are abundant in the outer root sheath of the hair follicle.

By chemical methods the glycogen content of the skin of the rabbit was

found to be about 0·1 p.c. (Calvery, Draize, and Land, 1946), and this glycogen, according to histological findings, would be concentrated in the lower part of the outer root sheath. Further evidence has now been adduced that these carminophilic granules in the root sheath may reasonably contain considerable amounts of glycogen. At present the origin of this glycogen is only a subject for speculation. For example, it may be that there is an accumulation of glycogen obtained from split products of protoplasmic constituents which become available in the process of epidermal cornification. Thus, some of the salvaged glycogen stored in the root sheath may find its way back into the circulation while an amount varying from species to species goes into the make-up of the hair. It is of interest to note that the rabbit, which has so far shown the smallest amount of carminophilic material in the root sheath by histological methods, was found to have by far the largest amount of glycogen in the hair using chemical procedures.

The periodic acid-fuchsin sulphite technique, more so than the carmine stain, slightly colours purple the surface and the interior of the non-medullated fibres of man and sheep. The cells of the medulla of phalanger and rabbit fibres contain areas which stain deep purple. However, it is not possible to remove the fuchsinophilic substance present by repeated extraction with boiling water, and therefore it may be assumed that some form of compound containing the adjacent glycol grouping is not water soluble. This might indicate a difference between the chemical composition of the cells of the medulla and the cortex.

SUMMARY.

In man, sheep, rabbit and phalanger, granules were observed in the outer root sheath which stained selectively with carmine and after periodic acid treatment, with fuchsin sulphite. According to chemical and histological evidence, these granules may contain glycogen. Fuchsinophilic areas in the cells of medullated hairs do not represent the glycogen extracted by water from these hairs.

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